COMPARATIVE ANALYSIS OF WINE TANNINS FROM PINOT NOIR GRAPES

by

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Finally, I dedicate this thesis to my Lord and Saviour Jesus Christ who encouraged me to undertake the entire research investigation with his own words:

"Everyone brings out the choice wine first and then the cheaper wine after the guests have had too much to drink; but you have saved the best until now." *John 2:10*

ABSTRACT

This thesis focuses on the sources of tannin in Pinot Noir grapes. Tannins contribute to colour stability and mouth-feel in wine. Some tannins are more beneficial to the quality of red wines than others. Achieving the correct balance is particularly difficult in Pinot Noir wines due to their unusual polyphenolic profile. In practical terms the thesis considered the following questions: How do we know from which berry tissues wine tannins are extracted? Are some tannins more desirable than others from a wine composition and quality viewpoint? How can we get more of the desirable tannins into Pinot noir wines?

The study sought to gain a better understanding of the types of phenolic compounds that influence wine quality in terms of colour stability. Phenolic compounds are mainly derived from the skin and seed of the grapes. However the nature and proportion of phenolic compounds that are contributed by each tissue type, and ultimately become incorporated into the wine, was largely unknown prior to this study.

The trials reported in this thesis used micro-vinification vessels ranging in size from 0.25 to 20 L according to the detail of the experiment under investigation and the analyses applied. Maceration techniques that are practiced commercially were compared and showed that some of these are better suited to the production of Pinot Noir table wines than are others.

To ascertain why this might be so, the research went on to examine the specific role of individual berry tissues in determining the outcome of the phenolic composition of the wine. The value of each tissue type was accentuated by providing a surplus or a deficit of each tissue type in the grape musts. The research examined the specific role of individual berry tissues in determining the outcome of the phenolic composition of the wine. A

novel maceration technique (Accentuated Cut Edges or ACE) that improved the extraction of colour pigments from grape skin was examined in detail and strong correlations between the phenolic profile of the wine and its sensory attributes were identified.

Finally, the spectral assessment of wine phenolic parameters from 15 individual experiments recognised six wavelengths by which phenolic components from skin and seed tissues could be distinguished. Using an existing rapid spectral assay, an index of phenolic quality was developed which has the potential to inform fermentation management strategies based on qualitative measures taken as the fermentation progresses.

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COMPARATIVE ANALYSIS OF WINE TANNINS FROM PINOT NOIR GRAPES

GENERAL INTRODUCTION

Pinot Noir is the premier red grape variety grown in Tasmania. This study was undertaken in light of the acknowledged complexities involved in making a high quality Pinot Noir table wine particularly in relation to the phenolic composition of the wine.

Pinot Noir is one of the most difficult grape varieties to grow requiring optimum growing conditions that include warm days (17 to 21^{o}C) consistently supported by cool evenings (6 to 9^{o}C). There are more than 1000 registered clones of Pinot showing diverse berry colour, yield, organoleptic qualities, leaf shape and cluster size (Robinson et al., 2012). Such diversity contributes to the challenges of making consistently high quality Pinot Noir table wines, as the various clones may behave differently under similar winemaking conditions. Further to this, many vineyards are planted with several different clones of Pinot Noir, adding to the range of responses shown by the fruit to the maceration technique selected during the winemaking process. In this thesis, the clone identification and fruit composition of Pinot Noir grapes has been specified where known for each of the experiments described.

1.1 PINOT NOIR VINIFICATION

The pigments (anthocyanins) and anthocyanin-tannin adducts contribute to the colour intensity, hue and colour stability of the wine (Bautista-Ortin et al., 2007, Bautista-Ortín et al., 2005). Both the pigments and skin tannins are located in the outer cell layers of the skin. Most red wine varieties have from six to ten skin cell layers, however Pinot Noir grapes have only two skin cell layers (Lecas and Brillouet, 1994). As a result Pinot Noir wines are paler in colour than the majority of red wines and the wine style is typically a lighter-bodied, fruit-prominent red wine tasting of red fruits like cherries, raspberries and strawberries (Hanson, 2014). As Pinot Noir wines age, the flavours and aromas become

more complex, developing earthy notes like mushrooms and decaying leaves (Hanson, 2014).

The anatomy of a red grape distinguishes the three major components of the berry: skin, pulp and seeds (Figure 1.1). Tannins are phenolic compounds and those extracted from grape skins are considered to be more important in the development of wine flavour than are those extracted from seeds (Cortell et al., 2005). Tannins are also extracted from grape stalks if they are included in the must (Robinson, 2006).

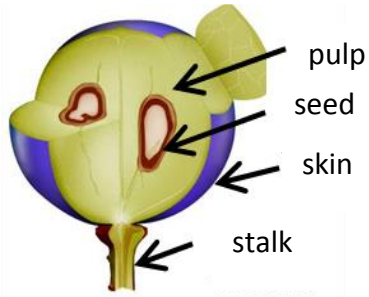


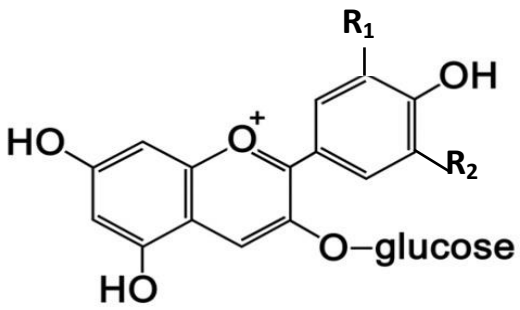
Figure 0.1. Tissues of the grape berry (Coombe, 1987)

The thin skins of *Vitis vinifera* cv. Pinot Noir grapes cause the ratio of skin tannins to seed tannins in the grapes to be low (Mattivi et al., 2009). These researchers isolated skin and seed tannins and reported that the ratio of skin to seed flavanols in Pinot Noir grapes was 1 to 63, while in Cabernet Sauvignon the ratio was 1 to 11 and in Shiraz 1 to 7.5. Subsequently, the wine quality for Pinot Noir has been strongly correlated to the skin tannin content of the grapes at harvest and the extraction of skin tannins together with colour pigments during winemaking (Neves et al., 2010). The term wine quality refers not only to the appearance, aroma, flavour and mouth feel of the wine, but also to its longevity.

This thesis focuses on colour stability of the wine as influenced by extraction of colour and tannin from the grapes and their interactions in the wine matrix.

1.2 GRAPE POLYPHENOLS

Tannins and anthocyanins belong to the group of naturally occurring plant-derived polyphenolic substances called flavonoids which affect the colour, ageing ability and texture of the wine. Some tannins are perceived as bitter while others cause a tactile dry sensation in the mouth (Somers, 1971, Kennedy et al., 2002, Vidal et al., 2004b, Ducasse et al., 2010a, Mercurio et al., 2010, McRae and Kennedy, 2011). Anthocyanins develop during veraison and are responsible for the change in colour of the skin from green through red to deep purple; they are found in the outer layers of skins cells (Kennedy and Cortell, 2004).



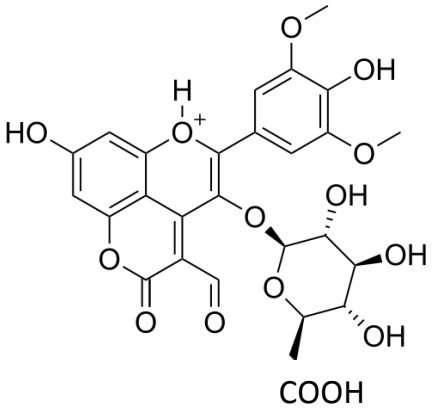
R_1, R_2 = H, OH, OCH_3

Figure 0.2 Basic structure of an anthocyanin molecule

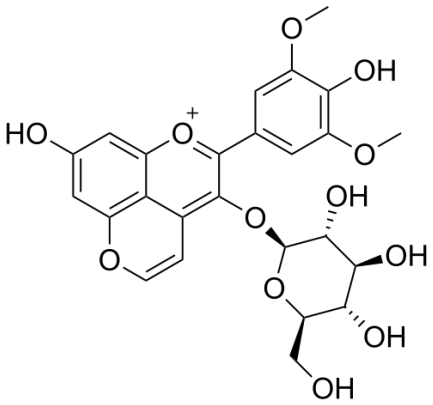
In wine solutions the different forms of anthocyanins are in equilibria according to their structure, the pH and the concentration of various co-factors in the wine matrix. The flavylium cation that occurs at low pH is red in colour, the quinoidal base becomes more

prevalent as the pH rises and is blue-purple, while both the carbinol and chalcone forms are colourless (Scollary, 2010).

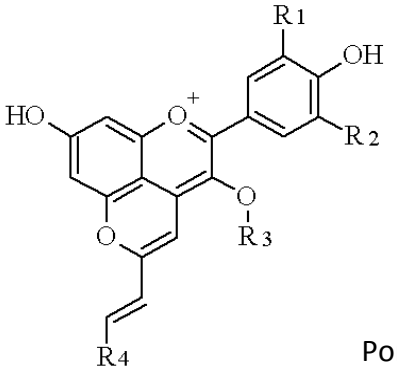
During the process of winemaking anthocyanins can undergo a range of reactions: they form self-associations or become co-pigmented with other low molecular weight compounds such as acetaldehyde, pyruvate, flavan-3-ol (Fulcrand et al., 1998, Romero and Bakker, 1999, Cheynier et al., 2006) and vinylphenol (Cameira-dos-Santos et al., 1996), to create an anthocyanin-derived pigment family, called pyranoanthocyanins. The most abundant of the pyranoanthocyanins are the vitisins and portisins (Figure 1.3).



Vitisin A



Vitisin B



R_1 = O-CH_3

R_2 = O-CH_3, OH or H

R_3 = glucose or acetylglucose

Portisin

Figure 0.3. Chemical structure of Vitisins and Portisins

These pigments are more stable when subjected to pH changes and bleaching by SO_2 than are anthocyanin monomers (FranciaAricha et al., 1997, Bakker and Timberlake, 1997, Romero and Bakker, 1999). During wine ageing, the vitisins undergo a progressive shift from the red colour of young wines to a more orange colour in older wines, while the portisins have blue-purple hue and are more resistant to discolouration as the wine ages (Marquez et al., 2013). Consequently their formation has a significant effect on wine colour stability (Sternad Lemut et al., 2013). A review by Scollary (2010) indicates that the contribution of copigmentation to wine colour is a controversial topic, but it is generally accepted that a decrease in copigmentation occurs with wine age, although the extent of the decrease may be variety dependent. For example, some reports suggest that the colour contribution of copigmented entities can decline from 45% to 0% in wines that are less than 10 months old (Hermosin et al., 2005). Yet others have detected copigmented entities in wines after one and two years (Darius-Martin et al., 2007, Lorenzo et al., 2005).

Pinot Noir wine grapes are unique in that they contain no acylated or coumarylated anthocyanins (He et al., 2012b, Heazlewood et al., 2006). As a consequence, self-association of anthocyanins by molecular stacking (hydrogen bonding) and copigmentation in Pinot Noir wines occurs to a lesser extent than in wines made from other varieties.

Anthocyanins and their derivatives (Figures 1.2 and 1.3) and tannins (Figures 1.4 to 1.7) have structural similarities and during the winemaking process, some of the tannins extracted from the grapes bind with anthocyanins to form stable colour compounds (Kennedy and Hayasaka, 2004) while others form polymeric molecules of varying mass (del Rio and Kennedy, 2006). Both types of polymeric compounds affect the sensory components that characterise the wine style, consequently the management of

anthocyanins and tannins in the winemaking process is a key component in ensuring red wine quality.

Note: The analytical procedure used throughout this research investigation to measure anthocyanin concentration, consisted of a single absorbance measure at 520 nm in acidified wine solution. The measure detects free anthocyanin and makes no distinction between the types of anthocyanin, consequently they have not been discussed in further detail.

In acidified solution (pH 1.0) all anthocyanins that are not bound in stable co-pigmented complexes are converted to the cationic form which is red in colour and can be detected at 520 nm. This value is considered relative to the absorbance of the wine sample in model wine solution (pH 3.4) containing 0.375% sodium metabisulphite and consequently gives an estimate of the concentration of anthocyanin that has not been stabilised.

1.3 TYPES OF TANNIN

The natural tannins found in grapes (proanthocyanidins) comprise a large class of polyphenols, in which flavan-3-ol molecules polymerise to form the larger proanthocyanidin molecules. Flavan-3-ol molecules are characterised by the oxygen heterocyclic ring system with an hydroxyl group at the 3 position (Figure 1.4).

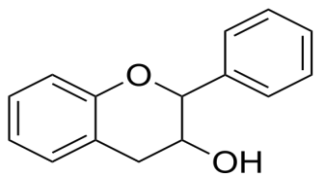
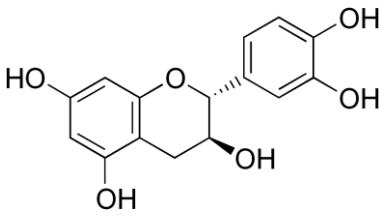
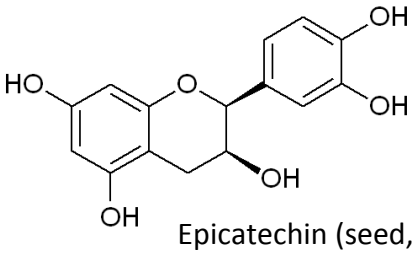


Figure 0.4. Basic structure of a flavan-3-ol molecule

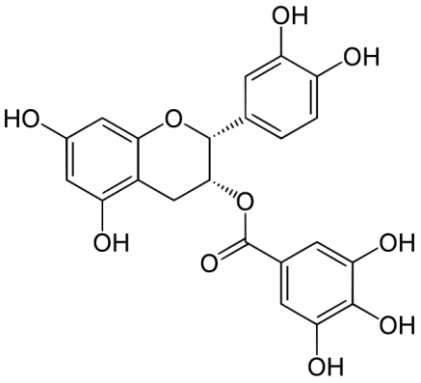
Grape seed extracts contain three monomers (catechin, epicatechin and epicatechin gallate) and procyanidin oligomers, whereas grape skin extracts contain four monomers: catechin, epicatechin, gallocatechin and epigallocatechin (Mattivi et al., 2009). Their chemical structure is shown in Figure 1.5. During the course of this investigation, the association of these monomers with specific skin and seed tissues provided an opportunity to differentiate skin and seed derived phenolic components during fermentation.



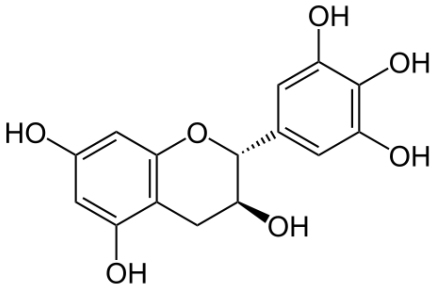
catechin (seed, skin)



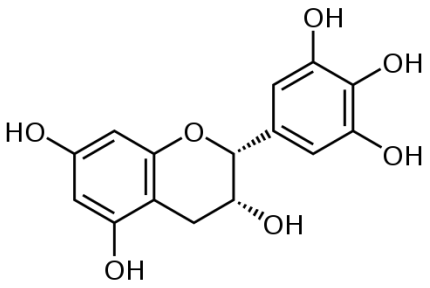
Epicatechin (seed, skin)



(+)-epicatechin gallate (seed)



(+)-gallocatechin (skin)



(+)-epigallocatechin (skin)

Figure 0.5 Chemical structure of grape flavan-3-ol monomers

Whole grape extracts are rich in monomers and small oligomers (mean degree of polymerization, less than 8). The simplest proanthocyanidins are oligomeric proanthocyanidins with 2 to 4 linked flavan-3-ol molecules as well as procyanidin oligomers made up of epicatechin subunits and prodelphinidin oligomers made up of gallocatechin subunits (Mattivi et al., 2009) (Figure 1.6).

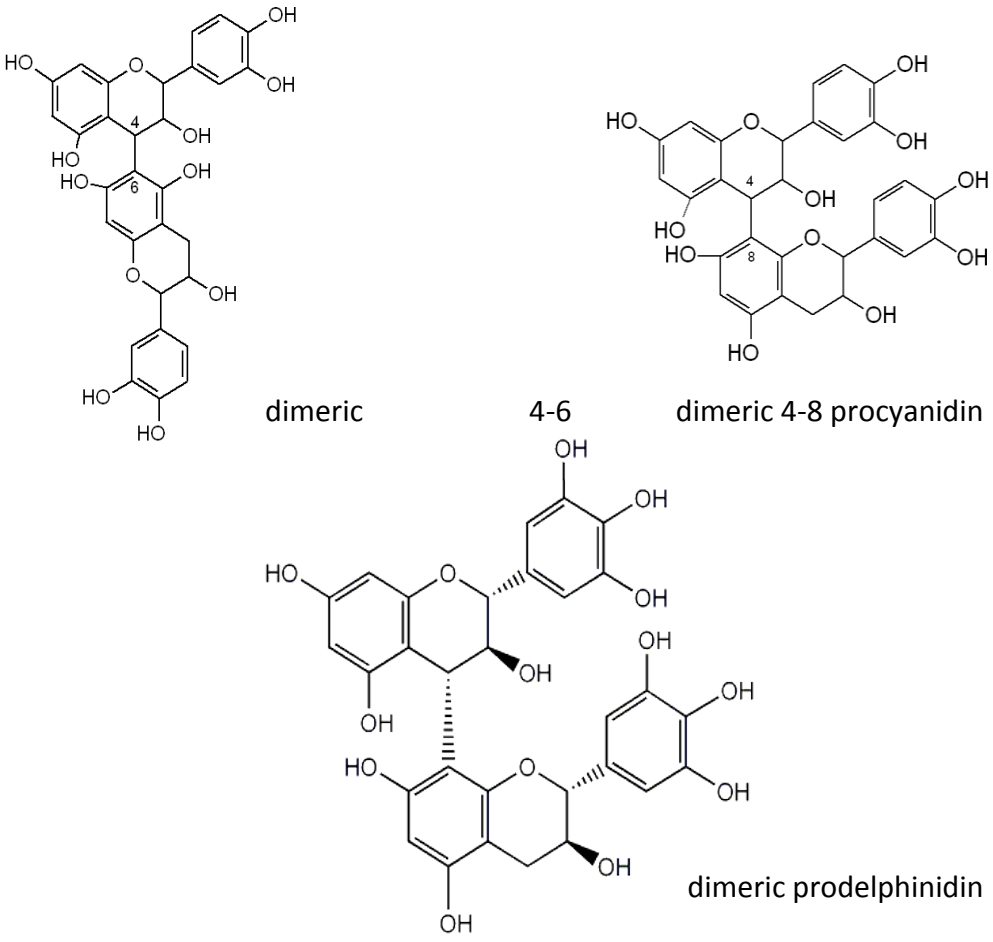
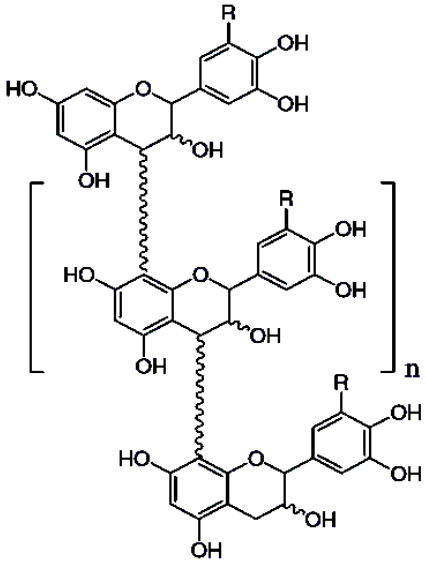


Figure 0.6 Chemical structure of simple proanthocyanidin oligomers

Subsequently larger tannins which have the same polymeric building blocks are described according to their mean degree of polymerisation (Figure 1.7).



Condensed tannin polymer
R = H, procyanidin
R = OH, prodelphinidin

Figure 0.7. Chemical structure of a polymeric proanthocyaninidin "condensed tannin"

Acetaldehyde produced either by the oxidation of ethanol or is liberated by yeast during fermentation, not only forms ethyl bridges between anthocyanins and flavan-3-ols, but between proanthocyandins trimers in the formation of condensed tannins. The reaction of tannins and anthocyanins with the phenolic compound catechin creates yet another class of tannins known as "pigmented tannins" which influence the colour of red wine and are resistant to sulphur dioxide bleaching (Kennedy and Hayasaka, 2004). As these are the most prevalent form of stable pigment in Pinot Noir wines, they have been given more detailed consideration in this thesis than other forms of stable pigment. In the chapters which follow, the term "tannins" will generally be used to describe tannins found in grapes as well as the tannins present in wine; where more detailed discussion of tannin subunits is required, the term proanthocyanidins will be used.

1.4 MACERATION TECHNIQUES

To counter the challenges presented by the grape cultivar, a range of winemaking procedures (maceration techniques) are employed which affect the contact of grape juice

and fermenting wine with the solid part of the grape must (pomace). Maceration techniques aim to optimise extraction of anthocyanins and tannins from the grapes and include cold soaking, SO_2 addition, thermovinification, grape or must freezing, pectolytic enzyme addition, carbonic maceration and extended maceration (Spranger et al., 1998, Yokotsuka et al., 2000, Sacchi et al., 2005, Villaño et al., 2006).

While anthocyanins are extracted in the early stages of maceration and reach their maximum amount before the start of alcoholic fermentation, tannins are extracted later in the fermentation and continue to increase in concentration until the end of fermentation (Spranger et al., 1998, Ribéreau-Gayon, 1982). Recent studies have demonstrated that it is not necessarily the increase in alcohol concentration that causes tannins to be extracted later in the fermentation as once thought, but the breakdown of the polysaccharide structure of pulp tissues and the loss of seed coat integrity (Watson et al., 2000, Sacchi et al., 2005, Gambuti et al., 2009) that promotes seed extraction.

1.5 EXOGENOUS TANNINS

The addition of oenological tannins to grape must is another means by which wine colour and its stability can be improved (Soto Vazquez et al., 2010, Bautista-Ortin et al., 2007, Bautista-Ortín et al., 2005, Gao et al., 1997). Historically, oenological tannins have not been known to assist in the extraction of anthocyanins from grapes, however they have been reported to provide a valuable contribution to the formation of polymeric pigments (Bautista-Ortín et al., 2005, Gao et al., 1997, Romero and Bakker, 2001). Caution is advised when using commercial oenological tannins as in some instances the desired effects of improved wine colour and colour stability can be reversed (Bautista-Ortin et al., 2007, Bautista-Ortín et al., 2005). The variations in the response of wines to oenological tannins

have been attributed to the many different preparations of oenological tannins and consequently, to their composition (He et al., 2012a).

1.6 PHENOLIC ANALYSIS OF GRAPES AND WINE

Analytical tools for the measurement of tannin concentration in grapes and wines have traditionally included methods relying on colorimetric endpoints, high-performance liquid chromatography (HPLC), depolymerization with acid in the presence of a nucleophile and precipitation with proteins or methyl cellulose (Herderich and Smith, 2005, Huemmer and Schreier, 2008). While research laboratories may use these analytical tools, uptake by the wine industry is generally constrained by time, technical knowledge and expense. By contrast, simple methods for the analytical determination of phenolic compounds associated with wine colour pigments have been available for forty years (Somers and Evans, 1974). More recently, spectroscopy combined with chemometrics has been used to measure grape and wine tannin and anthocyanins using visible–near-infrared (Vis-NIR) (Cozzolino et al., 2004a, Cozzolino et al., 2004b, Dambergs et al., 2003, Cozzolino et al., 2008) and mid-infrared (mid-IR) methods (Jensen et al., 2008, Patz et al., 2004, Fernandez and Agosin, 2007, Fernandez et al., 2007). Within the last decade, spectroscopy and chemometrics have also been adopted for the analysis of key grape and wine parameters such as alcohol, sugars, total phenols, organic acids, SO_2 and glycerol (Dambergs et al., 2004, Bauer et al., 2008, Cozzolino and Dambergs, 2010). The recent development of a technique to measure methyl cellulose precipitable tannins using ultraviolet spectroscopy and chemometrics (Dambergs et al., 2012) has provided a simple and rapid method of analysis of grape and wine tannins that requires a basic bench top UV-VIS spectrophotometer.

Despite these recent advances, there remains no simple method for differentiating seed and skin tannins in grapes and wine, with phloroglucinolysis and thiolysis currently the main methods used for this purpose (Torres and Lozano, 2001, Jorgensen et al., 2004). These analytical methods require not only significant expense, but sophisticated equipment and technological expertise. In order to address this gap in existing knowledge, this thesis used the method for rapid tannin analysis (Dambergs et al., 2012b) in conjunction with the modified Somers assay for wine colour (Mercurio et al., 2007) to evaluate the phenolic composition of the developing wine.

During 15 experiments conducted over 3 years, more than 3,000 samples of fermenting and maturing wine were analysed, with the aim of differentiating the contribution of skin and seed tissues to the phenolic profiles of Pinot Noir wines. The practical application of this knowledge was to develop a technique that will assist with the management of vinification methodology in commercial wineries.

1.7 MAIN OBJECTIVES

To optimise the extraction of beneficial tannins in red wine made from Pinot noir grapes a sequence of six studies were conducted:

- Determine the relative contribution of tannins from grape berry tissues;
- Investigate winemaking techniques that promote the extraction of preferred tannins;
- Emphasise the influence on wine colour stability of desirable tannins;
- Reduce the influence of undesirable tannins;
- Develop a novel maceration technique to optimise the extraction of grape phenolic compounds that augment the stable pigment content of the wine to improve wine quality;

- Describe a practical method of estimating phenolic quality of developing wine.

The following section outlines the objectives of each chapter.

Chapter 2 objective:

Determine whether the size and shape of the fermentation vessel has an impact on vinification outcomes with regard to the structure and quality of the wine. The development and evaluation of ultra-microvinification techniques at the northern Tasmania research laboratory has made possible fermentation experiments which could not be conducted on a larger scale.

Experiments of Chapter 2 scrutinised the response of Pinot Noir wine phenolic composition to different fermentation volumes. Some of the experiments described in subsequent chapters involved the use of fermentation vessels that were either small (1.5 L) or very small (250 mL), as they required intricate and time consuming must preparation. Others were large enough and of more conventional volume (15 L or larger) to provide sufficient volume of wine for replicated panel sensory analysis and industry-wide tastings. So the trials described in chapter 2 were conducted in fermentation vessels that covered the must weight range from 0.2 to 10 kg. In some instances it was useful to compare the results of trials conducted in 250 mL fermenters with those conducted in 1.5 L fermenters; in each case a control treatment was conducted in both vessel sizes.

Chapter 3 objective:

Assess the phenolic responses of a uniform parcel of fruit to six different maceration techniques that are practised in commercial wineries: de-stem and crush, cold soak, pectolytic enzyme addition, extended maceration, thermo-vinification, freeze-thaw.

The trial used a single vessel size (1.5 L) and highlighted the differences in the phenolic composition of wines at bottling and at 30 months bottle age.

Chapter 4 objectives:

Determine the tannin concentration in each berry tissue and distinguish the contribution of each grape tissue to the phenolic composition of the wine.

Experiments explored the role of different berry tissues, firstly by determining the total tannin content of isolated berry tissues extractable in 50% (v/v) ethanol, and secondly by fermenting isolated grape tissues in juice pressed from the same parcel of fruit. In these experiments, one or more berry tissues were omitted or doubled in the must to emphasize the contribution of each tissue type to the phenolic profile of the wine as it aged.

Chapter 5 objectives:

Evaluate common winery by-products as a source of grape tannin, that might address the phenolic imbalance inherent in Pinot Noir grapes and compare a commercially available skin tannin supplement with local sources of grape phenolic compounds.

The option of using readily accessible sources of grape phenolics that are otherwise discarded from commercial wineries was explored to ascertain if they might complement the phenolic profile of Pinot Noir wines. Grape tissues from Pinot Noir, Pinot Gris and Chardonnay cultivars of *Vitis Vinifera,* were compared with a commercial skin tannin supplement as exogenous sources of grape phenolics.

Chapter 6 objectives:

Assess the effect of seed tannins on wine quality; compare seed tannin from winery by-products with commercial tannin supplements and ascertain if the removal of seeds during fermentation can be justified.

The experiments highlighted in greater detail the impact of seed tannins on the phenolic composition of Pinot Noir wine, by supplementing or reducing the amount of seeds or seed tannin in the grape must. Histochemical studies of the seeds sampled during fermentation provided information on the location and rate of extraction of grape seed tannins.

Chapter 7 objectives:

Evaluate mechanisms that promote tannin extraction; differentiate methods for skin and seed tannin extraction and determine the influence of smaller skin particle size on the phenolic and sensory attributes of the wine.

Sequential experiments outlined the evolution of an innovative maceration procedure which reduced particle size of the grape skins, consequently extracting more colour pigment and tannin from the skins whilst leaving the seeds intact. The acronym ACE "Accentuated cut Edges" has been used to describe this novel technique.

Chapter 8 objectives:

Compile and contrast the spectral data from experiments conducted over the 3 years of the research; develop an analytical tool to monitor phenolic content during winemaking using laboratory equipment that is readily available in most wineries and explore a decision making protocol that may assist commercial wineries to direct the choice of maceration procedures which optimise wine quality outcomes for a parcel of fruit at any one time.

The findings were used to identify signature wavelengths that differentiated phenolic compounds derived from either grape skin or seed tissues. An algorithm was developed to provide an index of phenolic quality based on the balance between skin and seed phenolic compounds present in samples of both fermenting and finished wines.

1.8 STRUCTURE OF THESIS

This thesis follows the structure proposed by the University of Tasmania which encourages the publication of results in the scientific literature. Consequently, most chapters are organised like a scientific paper, and are able to stand as an independent contribution. Hence, there is some repetition between chapters, especially in introductory remarks. All references have been placed the end of the thesis to avoid repetition. Ensuing publications will have co-authors however their contribution to each chapter is not specifically mentioned in the thesis.

There are some exceptions: Chapter 1 is introductory, and is designed to serve the remaining thesis, not publication per se. Similarly, Chapter 9 provides concluding remarks and recommendations for future research including application of the new technologies. All chapter references with the body of the thesis refer to chapters of this thesis.

2

FERMENTATION VOLUME STUDIES FOR PINOT NOIR EXPERIMENTATION

This chapter has been prepared as a research note entitled:

'Fermentation Volume Studies for Red Wine Experimentation'

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2.1 Abstract

Vinification is often use to evaluate changes to viticultural and oenological practices in research trials, however various constraints make standardised comparisons in commercial wineries difficult to achieve. A dedicated micro-winery facility in northern Tasmania used conventional 12 L volume ferments providing sufficient wine for both sensory and chemical analysis. From 2009 to 2013 much smaller fermentation vessels of 1.5 L and 250 mL were introduced, providing sufficient must volumes for chemical analysis only of phenolic components in the wine. A comparison was made of the phenolic attributes of Pinot Noir in a replicated trial must weights of 0.2, 1.0, and 10 kg fermented in vessels of volume 250 mL, 1.5 L and 20 L respectively. A single larger ferment conducted concurrently, used the same parcel of fruit at must weight 330 kg in a vessel with volume 780 L. At bottling, six weeks after the end of fermentation, there was no significant difference in the phenolic composition of wine made from grape musts with mass 0.2, 1.0 or 10 kilograms in the replicated trial and the results were consistent with those for the 330 kg ferment size.

2.2 Introduction

Trials involving viticultural practices or winemaking methodologies need to be replicated in order to enable valid statistical analyses. Such conditions are difficult to achieve in commercial wineries due to the limitations imposed by production logistics, fermenter size and expense. Microvinification overcomes these problems by using small and uniform fruit

volumes and inexpensive fermentation and storage vessels together with temperature controlled rooms. Such techniques were developed in Europe, for example at Geisenheim Grape Breeding Institute Rheingau, Germany, for evaluation of new grape varieties (Becker, 1968) and were subsequently adopted in Australian grape and wine research (Becker and Kerridge, 1972, Antcliff and Kerridge, 1975). Microvinfication is now widely used for evaluation of viticultural and enological research trials in Australia and elsewhere, and its use has recently (20 January 2014) been reported in "approximately 999 citations (http://scholar.google.com.au/scholar)" using search words 'microvinification + wine + grapes'.

In 2005, a small-scale 'micro-winery' facility was developed at Kayena, Tasmania, Australia to evaluate the vinification responses to viticultural trials. In 2009, trials were extended to include oenology variables. Initially red wine ferments were carried out in 20 L food grade plastic buckets using 10 to 15 kg musts, and produced sufficient wine for chemical analysis and sensory evaluation. For the majority of experiments only chemical analysis was required to determine the outcome of the treatments applied. Subsequently smaller fermentation vessels, such as 1.2 L cylindrical glass jars and 1.5 L Bodum® coffee plungers were employed which used 700 g and 1 kg of must respectively, providing sufficient wine to take repeated samples for phenolic analyses (Dambergs and Sparrow, 2011, Carew et al., 2012, Carew et al., 2013). More recently, even smaller fermentation vessels (250 mL cylindrical polycarbonate jars with screw caps) have been used for Pinot Noir wine trials (Smart et al., 2012, Sparrow et al., 2013a). As little as 1.5 mL provides sufficient sample volume for phenolic analysis using ultraviolet-visible spectroscopy with chemometrics as described by (Dambergs et al., 2012b). This economical and demonstrably reproducible method of microvinification has been used to rapidly assess differences between parcels of

fruit and the effects of winemaking additives and processes. However the extension of outcomes from small-scale winemaking trials to commercial practice sometimes raise concern for oenologists, for example, the possible influence of must temperature in fermenters of different surface to volume ratio (Hornsey, 2007). While this study was not replicated at larger sizes, it compared the phenolic composition of wines made from a homogenous parcel of fruit with must weights 0.2, 1.0 and 10 kg, fermenter volumes of 250 mL, 1.5 L and 20 L and must surface to volume ratios of 0.15, 0.09 and 0.04. A larger ferment was conducted concurrently but without replication, using the same parcel of fruit in a fermentation vessel with must weight 330 kg and surface to volume ratio of 0.03.

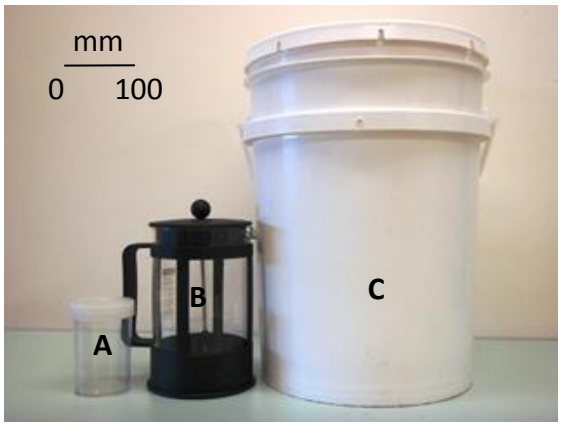
2.3 Materials and Methods

2.3.1. Grape sampling and replication

Grapes of *Vitis vinifera* cv. Pinot Noir clone D5V12, from drip irrigated vines, trained to vertical shoot positioning, were harvested from a 14 year old vineyard at Kayena, northern Tasmania in April 2013. Fruit from the vines of two adjacent and uniform rows were hand harvested into 20 L buckets. Fifty kilograms of grapes were transported to the nearby micro-winery where individual bunches were randomly allocated to four, 15 kg replicates for winemaking in vessels of different size. For each of the replicates, 200 g of berries were frozen at 20 ^{o}C and stored for grape colour and tannin analyses, and a further 100 berries were hand-crushed and the juice drained for fruit composition analyses. The remaining 330 kg of grapes were processed at the commercial winery on site.

2.3.2. Treatment preparation

For each of the replicates grapes were de-stemmed and three treatments applied: (1) 200 grams of randomly-selected berries were crushed by hand in a sealed plastic bag and the contents poured into a 250 mL polycarbonate jar; (2) 1 kg of berries were crushed by hand in a sealed plastic bag and placed in a 1.5L Bodum® coffee plunger; (3) 12 kg of grapes were de-stemmed and crushed in a Marchisio Grape Crusher/De-stemmer (1000 to 1500 kg/h) and placed in a 20 L food grade plastic bucket. The 330 kg of grapes was de-stemmed using a Bucher Vaslin Delta E4 Series Crusher/De-stemmer (25 to 30 t/h) at the commercial winery and placed in a 0.5 tonne square, food grade plastic, fruit picking bin. The fermentation vessels are shown in Figure 1.



A = cylindrical polycarbonate jar;

B = 1.5 L Bodum® coffee plunger;

C = 20 L food-grade plastic bucket.

D= 780 L plastic fruit-picking bin

Must weight A = 0.2 kg; B = 1 kg; C = 10 kg; D = 330 kg;

Must SA:V A = 0.15, B = 0.09; C = 0.04; D=0.03.

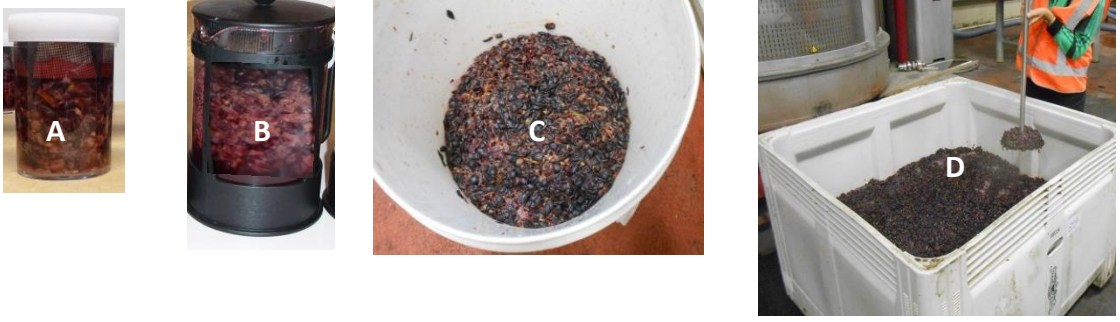


Figure 2.8 Fermentation vessels

2.3.3. Grape composition analysis

Total soluble solids in the grape juice (°Brix) were measured using a hand-held refractometer, the pH of the juice was measured using a Metrohm pH meter/autotitrator and titratable acidity was determined by titration with 0.333 M NaOH to an end point of pH 8.2 and reported as grams/litre tartaric acid.

2.3.4. Grape colour and tannin analysis

Frozen whole berries were thawed overnight at 4^{o}C and homogenised at 8000 rpm for 20 seconds in a Retsch Grindomix GM200 homogeniser, with an S25 N-18G dispersing element (Janke & Kunkel GmbH & Co, Germany) fixed with a floating lid. One gram of homogenate was subsequently extracted in acidified 50% (v/v) ethanol for the determination of grape colour (Iland et al., 2004) and tannin (Dambergs et al., 2012b).

2.3.5. Microvinification protocol

After preparing the musts, 50 mg/L SO_2 was added to each ferment in the form of potassium metabisulphite. Six hours later the must preparations had equilibrated to ambient temperature and were inoculated with 300 mg/L RC212 yeast solution and fermented at 27°C (±1°C). All fermentation vessels were covered with a loose fitting lid and plunged twice daily during fermentation to mimic cap management of the 330 kg ferment. On day three of the fermentation, 300 mg/L of diammonium phosphate was added to each ferment. After eight days, each fermenting must had proceeded to dryness which was confirmed at less than 2 g/L of residual sugar using Clinitest® reagent tablets (Bayer Australia Ltd.). The wine from the two smaller ferment sizes (0.2 and 1 kg) was then pressed by hand using a plunger with a mesh sieve (mesh size 1 mm x 1 mm) fixed at the base, while the 10 kg and 330 kg ferments were pressed in a flat-bed press at 200 kPa of

pressure. In each case enough pressure was applied to recover 60% (v/w) of the must weight that is, 120 mL, 0.6 L, 6.0 L and 220 L of wine respectively. Wine was stored in glass screw topped bottles at 4°C for 14 days, at which time it was racked under CO_2 cover and a further 80 mg/L SO_2 added. The wine was stored for a further 30 days at 12°C when it was racked a second time under CO_2 cover and bottled in either amber or green screw topped bottles. Six weeks after the end of fermentation, a 10 mL sample from each replicate and ferment size was taken for phenolic analysis.

2.3.6. Phenolic analysis by spectroscopy

Wine samples were clarified by centrifugation at 5,000 rpm for five minutes using an Eppendorf 5417C centrifuge with an Eppendorf F45-30-11 rotor (Crown Scientific, Australia). The phenolic composition of all samples were determined using the modification of the Somers assay described by Mercurio et al. (2007) with model wine buffer for colour analysis prepared using 5 g/L potassium hydrogen tartrate in 12% (v/v) ethanol. Assays were performed following a 1:10 dilution of wine in either model wine buffer containing 0.1% (v/v) acetaldehyde or model wine buffer containing 0.375% (w/v) sodium metabisulfite. Wine for the rapid tannin assay described by (Dambergs et al., 2012b) was diluted 1:50 with 1.0 M HCl.

The phenolic parameters quantified were total tannin (pigmented and non-pigmented tannins); total phenolics (coloured and non-coloured tannins and anthocyanins, and low molecular weight, non-pigmented phenolic compounds); free anthocyanin (unbound anthocyanins); non-bleachable pigments (resistant to bleaching in the presence of sulphur dioxide, they consist of either pigmented tannins in which one or more anthocyanin molecules have become bound to proanthocyanidins (condensed tannins) (Harbertson et al., 2003) or pyranoanthocyanins (Boulton, 2001, Cheynier et al., 2006). Colour density

(the degree of pigment saturation of the wine); and hue (the nature of wine colour - higher hue values appearing ruby-garnet, and lower values appearing more blue-purple). UV-Visible spectrophotometric analysis was conducted using a Thermo Genesys™ 10S UV-Vis Spectrophotometer. Samples were scanned in 10 mm cuvettes, at 2 nm intervals over the wavelength range 200-600 nm.

2.3.7.Statistical analysis

Statistical analyses were conducted using GenStat 64-bit Release 14.2 Copyright 2011, VSN International Ltd. The mean, standard deviation and coefficient of variation for fruit composition characters, the phenolic content of grapes and the individual phenolic analytes in wine samples for each treatment replicate (n=4) were calculated using 1–way ANOVA. For each phenolic character ANOVA was followed by post-hoc analysis using Tukey's 95% confidence test. The 330 kg ferment was unreplicated and excluded from statistical analyses.

2.4 RESULTS

Fruit composition

The composition of the grapes used in each trial was determined prior to fermentation and is described in Table 1. The fruit used for vinification was quite uniform; the co-efficient of variation (CV) was highest for berry weight at 7.7%; all other grape composition and phenolic characters had lower CVs with pH being the lowest at 1.3%.

Table 2.1 Fruit composition of *Vitis vinfera* cv. Pinot Noir Clone D5V12.

(mean, standard deviation (STDEV) and co-efficient of variation (CV); n=4)

Berry component	Mean ± STDEV	%CV
Berry Wt (g)	136.8 ± 10.5	7.7
Total Soluble Solids (^{o}Brix)	21.20 ± 0.49	2.3
pH	3.22 ± 0.04	1.3
Titratable acidity (g/L)[Ŧ]	7.70 ± 0.24	3.1
Anthocyanin (mg/g)	0.58 ± 0.03	5.9
Total tannin (mg/g)	6.87 ± 0.24	3. 6
Total phenolics (AU/g)	1.18 ± 0.08	7.0

[Ŧ] Titratable acidity expressed as grams of tartaric acid equivalents

2.3.1. Phenolic composition at bottling

The vessels used to ferment the musts are shown in Figure 2.1. The surface to volume ratio (SA:V) became smaller as the fermentation vessel size increased (Table 2.2). For each of the phenolic analytes assessed at bottling: total phenolics, tannin, colour density, total pigment, free anthocyanin, non-bleachable pigment (resistant to SO_2 bleaching) and percentage non-bleachable pigment, there was no correlation with either the must weight or the surface to volume ratio of the fermenting musts (0.2 kg, 1 kg, 10 kg musts or 330 kg). There was a correlation between wine hue and vessel size but this is likely to be attributed to the low level of variation (3%) between treatment replicates.

Table 2.2 Must sizes for ferments made in vessels of different capacity.

Vessel capacity (L)	0.25	1.5	20	780
Must weight (kg)	0.2	1	10	330
Must diameter (cm)	6.5	12	26	NA
Must height (cm)	6.5	11.3	24	31
Must surface area (cm^2)	33	113	531	10,000
Must volume (L)	0.22	1.28	12.7	310
Must surface:volume	0.15	0.09	0.04	0.03

NA, not applicable

Only wine hue showed a small (7%; P=0.04) but significant effect of ferment size, the 0.2 g ferment size (SA:V = 0.15) having a lower hue value than the 10 kg ferment size (SA:V = 0.04) (Table 2.3).

Table 2.3 Phenolic composition at bottling of Pinot Noir wine made in fermentation vessels of varying must capacity.

Must Size (kg)	0.2	1	10	⁋330	%CV	*P-value*
surface:volume ratio	0.15	0.09	0.04	0.03	(n=4 x size)	(0.05%)
Tannin (g/L)	0.23	0.22	0.33	0.22	30.9	0.14
Anthocyanin (g/L)	0.16	0.17	0.19	0.15	9.4	0.12
†NB pigment (AU)	0.32	0.32	0.32	0.30	12.4	0.94
Total pigment (AU)	8.54	8.86	9.88	7.91	9.3	0.14
% NB pigment	3.82	3.65	3.23	3.80	8.1	0.06
Total phenolics (AU)	24.6	24.3	28.1	24.0	7.4	0.06
Colour density (AU)	2.61	2.63	2.62	2.46	8.2	0.21
Hue (AU)	0.73 a	0.71 ab	0.68 b	0.81	3.0	0.04

For each ferment size 0.2, 1 and 10 kg, there were 4 replicates (n=4); †NB = non-bleachable pigment. For each phenolic parameter only means followed by a different letter were significantly different using Tukey's test ($p \leq 0.05$). ⁋Unreplicated and excluded from statistical analyses.

2.5 DISCUSSION

To our knowledge, trials to determine the effect of fermentation volumes have not been previously reported for wine grapes. However using phenolic analysis by spectroscopy and chemometrics (Mercurio et al., 2007, Dambergs et al., 2012b) a variety of significant treatment effects representing changes in either viticultural or enological practices have been evaluated for the smaller vessel volumes described here. These include yeast strain effects on wine quality, maceration techniques during vinification, and wine quality

responses to UV radiation and vine vigour (Dambergs and Sparrow, 2011, Song et al., 2015, Carew et al., 2012, Dambergs et al., 2012a, Carew et al., 2013b, Song et al., 2013).

This study demonstrates that for Pinot Noir grapes the phenolic composition of wine bottled six weeks after the completion of fermentation was independent of must sizes ranging from 0.2 to 10 kg in weight, for seven out of the eight phenolic characters assessed. The total tannin content of the wine showed the greatest coefficient of variation (31%) between replicates for the eight phenolic characters determined, which was greater than the CV (5-10%) found in other trials (Sparrow et al., 2013a). A possible explanation for the larger variation here, is the use of twice daily plunging for cap management rather than submerged cap fermentations as used in experiments which followed. With the exception of NP (CV 12.4%), the remaining phenolic characteristics consistently showed less than 10% variation the majority of which may be attributed to the 7% CV in the phenolic composition of the fruit (Table 2.1). The CV for wine hue was the lowest of the phenolic characters at 3% (P=0.04). Consequently, a minor difference in wine hue between the 0.2 kg must size and 10 kg must size was detected (Table 2.3).

In summary, micro- and ultra-micro vinification procedures were found to be a reproducible method for assessing the phenolic composition of wine made from musts over the range 0.2 to 10 kilograms..

The larger ferment size (330 kg) conducted concurrently using the same fruit source did not have the same strict conditions of fermentation temperature. However the values for each phenolic parameter in wines from this ferment size were within the range of those of the three smaller ferments (Table 2.3). This suggests that the results of the microvinification trial results may be extrapolated to larger ferment sizes, but such a conclusion needs further controlled comparisons. This investigation focused on the colour

stability of Pinot Noir wine, in addition potential differences in aroma and flavour characteristics of wines made in fermentation vessels with a range of surface area to volume ratios is also worth persuing.

2.6 CONCLUSION

The results of this study not only confirmed the benefits of microvinification as a research tool that can be modified to accommodate the number of progressive samples and the volume of finished wine required for analysis, but they also demonstrated that phenolic evaluation of the wine is independent of vessel volume over the must weight range from 200 grams to 10 kilograms and possibly larger.

2.7 SUBSEQUENT RESEARCH FOCUS

The fermentation size study demonstrated that the phenolic composition of wines made in ferment vessels ranging in size from 200 g to 10 kg was comparable. The musts sizes of subsequent experiments in this thesis were either small (200 g or 1.0 kg), and involved intricate must preparation or large enough to provide sufficient volume of wine for sensory analysis (10 kg). In some instances it was useful to compare the results of trials conducted with must weight 200 g with those having must weights of 1.0 kg, in each case a control treatment was conducted in both vessel sizes to ensure consistency was maintained.

3

RESPONSE OF PINOT NOIR WINE PHENOLICS TO MACERATION TREATMENT

This chapter is in preparation as a technical research article.

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3.1 ABSTRACT

Pinot Noir wine has an unusual phenolic profile which has implications for wine quality. Varying the maceration technique used during vinification can enhance the extraction and stabilisation of phenolic compounds in Pinot Noir wines. Six maceration treatments were compared in a replicated micro-vinification trial: no intervention (control), pectinase enzyme addition, microwave treatment, cold soaking, must freezing and extended maceration. Analysis of the wines at six and 30 months bottle age showed that the enzyme addition had little influence on wine phenolics. At six months bottle age, cold soaked treatment wines were no different to the control wines but by 30 months bottle age, cold soaked wines had significantly higher concentrations of pigment resistant to sulphite bleaching (non-bleachable pigment) than the control wines. Relative phenolic effects changed as the wines aged with extended maceration wines being significantly higher in non-bleachable pigment than all the other treatments at six months bottle age and significantly lower in free anthocyanin, suggesting advanced colour stabilisation. However, by 30 months bottle age, the non-bleachable pigment concentration of the extended maceration wine had declined by 30% suggesting that some of the non-bleachable pigment did not remain stable during storage. Wines made from grapes that had been frozen and thawed prior to fermentation were high in tannin relative to stable pigment at 30 months bottle age. With the exception of wines treated with pectinase enzyme, all treated wines demonstrated some difference in wine phenolic composition

relative to the control wines at 30 months bottle age. The study demonstrated that maceration is a valuable tool for winemakers in managing the phenolic character of Pinot Noir wine, and that both co-pigmentation and the polymerisation of anthocyanin and tannin to form non-bleachable pigment proceeds at different rates depending on the maceration technique. The influence of bottle age demonstrates that timing of analysis is important when judging the merit of various maceration techniques.

3.2 INTRODUCTION

The extraction and stabilisation of phenolic compounds is central to making good quality red wine (Peynaud, 1984). Phenolic compounds fall into two major classes – anthocyanins, which form the basis of red wine colour, and tannins, which influence textural properties of the wine and also form complexes with anthocyanins to produce stable colour pigments (polyphenolic pigmented tannins). Hydroxycinnamates, benzoic acids and flavonols are also involved in colour and flavour expression. The concentration of anthocyanin and tannin in wine has been correlated with measures of red wine quality such as wine scores at judging, and retail price (Cozzolino, et al. 2008, Holt, et al. 2008, Kassara and Kennedy 2011, (Ristic et al., 2010). While the term wine quality refers not only to the appearance, aroma, flavour and mouth feel of the wine, but also to its longevity, this investigation focuses on the intensity and stability of Pinot Noir wine colour.

Pinot Noir grapes tend to have a low concentration of anthocyanin per berry, and of the five types of anthocyanin found in Pinot Noir, all are of a non-acylated form (Heazlewood, et al. 2006, Mazza, et al. 1999). While the total quantity of tannin in Pinot Noir grapes is similar to that of other well studied varietals like Cabernet Sauvignon, Merlot and Shiraz, the tannin in Pinot Noir is disproportionately distributed (high in seeds, low in skins)

(Mattivi, et al. 2009, Kennedy 2008, Downey, et al. 2003). By contrast, wines made from Pinot Noir grapes are often low in tannin concentration, compared with other red wine varieties (Harbertson, et al. 2008) which may be attributed to the late extraction of seed tannins relative to skin tannins (Watson et al., 2000, Sacchi et al., 2005, Gambuti et al., 2009, Koyama et al., 2007a). In analysing the tannin concentration of 1350 commercial red wines Harbertson et al. (2008) found Pinot Noir to have approximately half the concentration of tannin (catechin equivalents) compared with Cabernet Sauvignon and Merlot. As a result of the unique distribution and phenolic composition of Pinot Noir grapes, winemakers look for alternative methods for optimising the extraction of these compounds.

One option available to winemakers is to vary the maceration approach applied before, during or immediately after alcoholic fermentation. Maceration processes are applied to grape must to control the release of phenolic compounds from berries into juice.

A review by Sacchi et al. (2005) identified six maceration practices that were effective in increasing phenolic concentration in red wine: fermentation temperature, thermovinification (to degrade cell elements and hasten phenolic diffusion); must freezing, saignée ('bleeding off'), pectolytic enzyme treatment (to degrade grape cellular elements) and extended maceration. In addition to these techniques, cold soaking is often used in the vinification of Pinot Noir; this technique is intended to enhance colour diffusion while simultaneously hindering the onset of fermentation. While most research confirms the effectiveness of thermovinification and extended maceration for enhancing phenolic extraction, there have been conflicting reports related to the use of freezing, enzyme addition and cold soak maceration treatments. Cold soaking has been described as having no effect, little lasting effect or a detrimental effect on wine colour, compared with

conventional fermentation (Joscelyne, 2009, Sacchi et al., 2005). However, others concluded that cold soaking increased the concentration of some phenolic compounds in red wine (Gil-Munoz et al., 2009, Reynolds et al., 2001, Busse-Valverde et al., 2010). A replicated trial comparing the impact on anthocyanin concentration in Shiraz wine of cold soaking and extended maceration concluded that untreated control wines at bottling were significantly lower in anthocyanin concentration than the cold soak and extended maceration treatments (370 mg/L, 416 mg/L, 444 mg/L respectively) (Reynolds et al., 2001). However the cold soak treatment applied in that study was relatively long (10 days); the concentration of polymeric pigment in the wine was not reported and the impact of bottle ageing was not examined. The use of macerating enzymes in Pinot Noir wine making examined by Parley et al. (2001) was shown to have a significant impact on the concentration of anthocyanin and polymeric pigment in wines; anthocyanin was low in enzyme treated wines while polymeric pigments were high, and those effects were maintained to 18 months bottle age. Other work reports that the effectiveness of maceration processes like cold soaking and enzyme addition vary by grape variety (Busse-Valverde et al., 2010) and by the enzyme preparation used (Wightman et al., 1997). The use of must freezing as a maceration method was compared with cold soaking, dry ice maceration and enzyme treatment for Cabernet Sauvignon and Syrah winemaking (Gil-Munoz et al., 2009). All these pre-fermentation maceration techniques were found to be effective for colour extraction, however the researchers pressed the wine off at the conclusion of alcoholic fermentation, so skin contact time for the five treatments ranged from 12 to 28 days. This means that the effects observed may simply have been due to greater or lesser opportunity for diffusion of phenolic components from the berry tissues, rather than the influence of the treatments applied.

The aim of this study was to compare the effect of six maceration treatments on the phenolic composition of Pinot Noir wine and to examine the relative response of the wines to bottle ageing. The first sample was taken at six months bottle age and represented the time at which winemakers would potentially make blending and price point decisions. The second sample taken at 30 months bottle age time and represented the time at which consumers are likely to drink the wine and evaluate its quality.

3.3 MATERIALS AND METHODS

3.3.1 Treatment preparation

30 kilograms of Pinot Noir grapes were harvested from a vineyard in Northern Tasmania, Australia at 12.5°Baume and pH 3.25. Grapes were sampled for fruit composition and then bunches were randomly allocated into 24 replicates, each approximately 1.1 kg. Four replicates were allocated to each of six maceration treatments. Prior to application of the maceration treatments, grapes from each replicate were crushed using an Enoitalia manual bench-top crusher, and de-stemmed by hand before the resulting must was decanted to a 1.5L Bodum™ coffee plunger for fermentation following the 'French Press' submerged cap method (Dambergs and Sparrow, 2011, Dambergs et al., 2012a). The maceration treatments for this trial were: (1) control, (2) pre-fermentation enzyme addition, (3) pre-fermentation microwave maceration, (4) pre-fermentation cold soak (4 days), (5) pre-fermentation freezing and thawing (3.5 days), and (6) post-fermentation extended maceration (4 days).

To each fermentation vessel 50 mg/L sulphur dioxide (SO_2) was added in the form of potassium metabisulfite solution. Control and extended maceration treatments were placed in a constant temperature room (27°C ±3°C) prior to inoculation. Enzyme treated

musts had 300 mg/kg of pectolytic enzyme Lafase HE (Lafazyme) added to the must prior to placement at 27°C ±3°C. The microwave treated must was heated to 70°C by microwave radiation, held at that temperature for one hour and cooled in an ice-bath for approximately 30 min (Carew et al., 2013a). Cold soak treated musts were covered with plastic film (Glad Wrap™) and stored for 4 days at 4°C prior to warming to ambient temperature (20°C) for 4 hours followed by a further 2 hours warming to 27°C (±3°C) in preparation for inoculation. Freeze-thaw treated musts were covered with plastic film (Glad Wrap™) and stored at minus 20°C for 3.5 days prior to thawing for 6 hours at ambient temperature (20°C) and warming at 27°C (±3°C).

3.3.2 Microvinification protocol

Approximately three hours after applying the control, enzyme, microwave and extended maceration treatments, those ferments were inoculated with the active dried yeast strain *Saccharomyces cerevisiae* EC1118 (Lallemand, Australia) that had been rehydrated according to the manufacturer's instructions. This yeast strain was selected for its tolerance to variations in temperature that were imposed on the fermenting musts. Cold soak and freeze-thaw treated musts were inoculated in the same way at day 4 of the experiment. This meant that control, enzyme and microwave treatments had 8 days of skin contact time prior to the completion of alcoholic fermentation, whereas cold soak, freeze-thaw and extended maceration treatments had 12 days of skin contact time.

All fermenters were incubated at 27°C (±3°C) and weighed regularly over the course of the 8 or 12 day ferment to track CO_2 loss (an indicator of the progress of fermentation). At day 3 after yeast inoculation, 60 mg/L of yeast assimilable nitrogen was added to each fermenter in the form of diammonium phosphate solution. Control, enzyme and microwave treatments were pressed off at day 8 and cold soak, freeze-thaw and extended

maceration treatment fermenters were pressed off at day 12. Pressing off was achieved by applying firm downward pressure on the Bodum™ coffee plunger to a specified mark on the vessel, after which wine was decanted into 375 mL green glass bottles closed with stelvin screw caps and incubated at 27°C (±3°C) for 12 hours to ensure fermentation was complete. Wines were tested for residual sugar using Clinitest™ tablets and all wines were found to be dry (≤2.5 g/L residual sugar). The wine was then cold stabilised for two weeks at 4°C to precipitate potassium hydrogen tartrate, and then racked into 250 mL Schott™ bottles under CO_2 cover and stabilised against microbial attack and oxidation by the addition of 80 mg/L sulphur dioxide (SO_2) in the form of potassium metabisulfite solution. After two weeks, wines were bottled under CO_2 cover into 25 mL amber glass bottles, sealed with polypropylene screw caps and stored for bottle ageing. Polypropylene screw caps have been reported to have oxygen ingress similar to a synthetic cork (Peck, 2014).

3.3.3 Phenolic analysis by spectroscopy

The phenolic composition of the wines was determined at six and 30 months bottle age using the modification of the Somers assay described previously (Mercurio et al., 2007) and rapid tannin analysis (Dambergs et al., 2012b). The phenolic parameters quantified were: total tannin (pigmented and non-pigmented tannins); total phenolics (coloured and non-coloured tannins and anthocyanins, and low molecular weight, non-pigmented phenolic compounds); free anthocyanin (unbound anthocyanins); non-bleachable pigments (resistant to bleaching in the presence of sulphur dioxide, they consist of either pigmented tannins in which one or more anthocyanin molecules have become bound to proanthocyanidins (condensed tannins) (Harbertson et al., 2003) or pyranoanthocyanins (Boulton, 2001, Cheynier et al., 2006)); colour density (the degree of pigment saturation of the wine); and hue (the nature of wine colour - higher hue values appearing ruby-garnet,

and lower values appearing more blue-purple). UV-Visible spectrophotometric analysis was conducted using a Thermo Genesys™ 10S UV-Vis Spectrophotometer. Samples were scanned in 10 mm cuvettes, at 2 nm intervals over the wavelength range 200-600 nm.

3.3.4 Statistical analysis

Means and standard deviation were calculated in Excel™ for phenolic concentration measures at six and 30 months bottle age, and for pH at 30 months bottle age. GenStat 64-bit Release 14.2 Copyright 2011, VSN International Ltd. was used to calculate one-way and two-way ANOVA to identify treatment effects on wine phenolics at six and 30 months bottle age, and Tukey's test was used for post-hoc analysis. In the following section, one-way ANOVA results are reported separately for each bottle age period as the periods represent two distinct points at which the phenolic quality of wines would be evaluated independently. Interactions between the maceration treatment and bottle age were analysed using two-way ANOVA and are reported after one-way ANOVA results in the following section as they are indicative of the relationship between the winemakers' decision point and wine consumption.

3.4 RESULTS

The maceration treatments applied in this study were associated with significant differences in phenolic concentration in wines, and the pattern of difference changed with bottle ageing from bottling (50 days after inoculation) to 30 months bottle age (960 days after inoculation). The phenolic characteristics of the wines prior to the commencement of bottle ageing (50 days after inoculation) are listed in Appendix 3.1

On the basis of the seven phenolic measures examined at six months bottle age, neither the enzyme nor cold soak treated wines were statistically distinguishable from control

wines (Table 3.1). At this time period the coefficient of variation across all treatments was 10% with the control, cold soak and freeze-thaw treatments showing the greatest variation (12.8%). Freeze-thaw and microwave treated wines had tannin concentrations two-fold higher than control wines, and had total phenolic concentrations that were 44% higher than control wines. The tannin concentration of extended maceration treated wine was 61% higher than control wine, but significantly lower (49%) than freeze-thaw and microwave treated wines. Microwave treated wine had the highest anthocyanin concentration of all treatments (89% higher than control), and extended maceration had the lowest anthocyanin concentration (2.5-fold lower than control). Extended maceration treatment wines were significantly higher (2.8-fold higher) in non-bleachable pigment concentration than all other treatments, suggesting advanced formation of stable pigments under this maceration treatment (Table 3.1). Across the treatments there was a small but significant range of hue values with microwave and extended maceration treatments having the lowest hue (more blue-purple colouration). At 30 months bottle age, the coefficient of variation for phenolic parameters averaged 14% across all treatments with the exception of the extended maceration treatments which had CV 9%. Control and enzyme treated wines were indistinguishable from each other on the basis of phenolic composition. Freeze-thaw treated wines had a higher concentration of both total phenolics and tannin than the control wines, (34% and 2.7-fold respectively) but had no greater concentration of non-bleachable pigment than the control. Cold soak and extended maceration wines were significantly higher (51%) than control wines in non-bleachable pigment, while microwave treated wines were significantly higher in all of the phenolic parameters examined relative to the control wines. Analysis of hue values showed that the majority of treated wines had

higher hue values at 30 months bottle age than at 6 months bottle age which was indicative of a shift to the red-orange colour range.

Interactions were observed between maceration treatment and bottle age for all seven of the phenolic parameters examined in this study (total phenolics P=0.003; total pigment P<0.001; anthocyanin P<0.001; SO_2 resistant (non-bleachable) pigment P<0.001; tannin P=0.009; colour density P<0.001; hue P=0.002). This meant that the relative impact of maceration treatments changed as the wines aged. The phenolic composition of the cold soak treated wines was indistinguishable from control wines at six months bottle age, but at 30 months these wines had a non-bleachable concentration that was 51% higher than control wines. The extended maceration treatment apparently developed SO_2 resistant pigment more rapidly than the other treatments being 2.8- fold higher for this measure at six months bottle age, however it was the only treatment in which SO_2 resistant pigment concentration declined (40%) by 30 months bottle age relative to six months bottle age (Table 3.1).

Table 3. Error! No text of specified style in document. **Pinot Noir wine phenolics at 240 and 960 days post-inoculation (mean; n=4) for six maceration treatments.**

Phenolic character	control	enzyme	micro-wave	cold-soak	freeze-thaw	extended maceration
	Wine at 240 days post-inoculation (6 months bottle age)					
Total phenolics (AU)	28.2 a	30.22 a	41.1 b	29.8 a	40.1 b	30.1 a
Total pigment (AU)	7.66 ab	7.71 b	13.9 c	7.82 b	9.43 b	5.61 a
Anthocyanin (mg/L)	133 b	131 b	252 c	138 b	169 b	52 a
NBP (AU)[Ŧ]	0.60 a	0.69 a	0.79 a	0.57 a	0.59 a	1.80 b
Tannin (g/L)	0.38 a	0.51 ab	0.88 c	0.47 ab	0.94 c	0.61 b
Colour density (AU)	2.91 a	3.23 a	4.31 b	4.58 b	3.07 a	4.58 b
Hue (AU)	0.86 a	0.8 ab	0.75 bc	0.81 ab	0.83 ab	0.69 c
	Wine at 960 days post-inoculation (30 months bottle age)					
Total phenolics (AU)	18.7 a	20.8 ab	25.5 c	20.0 a	25.0 bc	21.1 abc
Total pigment (AU)	1.47 a	1.85 ab	2.42 b	1.93 ab	1.90 ab	2.04 ab
Anthocyanin (mg/L)	1.16	ND	ND	ND	ND	ND
NBP (AU)[Ŧ]	0.85 a	1.14 ab	1.46 b	1.28 b	1.19 ab	1.29 b
Tannin (g/L)	0.15 a	0.32 ab	0.43 bc	0.33 ab	0.56 c	0.41 bc
Colour density (AU)	2.58 a	2.83 ab	3.71 b	2.82 ab	3.08 ab	2.71 a
Hue (AU)	1.39 a	1.10 abc	1.04 bc	0.91 bc	1.16 ab	0.85 c
pH	3.23 ab	3.17 a	3.46 bc	3.48 bc	3.49 bc	3.63 c

Significant differences in concentration for each parameter within the corresponding bottle age period (Tukey's Test $P \leq 0.05$) are represented by different letters. [Ŧ]NBP, Non-bleachable pigment; ND, below detection limit for the assay; NA, not applicable

3.5 DISCUSSION

3.5.1 Effect on wine phenolics

This study demonstrated that management of the phenolic profile of Pinot Noir wine can be significantly affected by the maceration treatment selected by the winemaker. The age at which bottled wine was evaluated for phenolic quality was of critical importance in the appraisal of the maceration techniques as the relative phenolic qualities of wines (as indicated by the phenolic measures examined in this study) changed between six and 30 months bottle age. For example, cold soaking appeared to have conferred no benefit compared with control treatment at six months bottle age, but at 30 months these wines had a greater proportion of stable colour (non-bleachable pigment) than control wines. Conversely, at six months bottle age the extended maceration treated wines had a significantly higher (2.8-fold) non-bleachable pigment concentration than all the other treatments. However by 30 months bottle age, the non-bleachable pigment concentration of extended maceration treated wines had declined and remained significantly higher than the control wine alone. Analysis of the species of phenolics that are extracted from grape solids into juice over the course of red wine fermentation has shown that skin-associated phenolics are extracted in the early part of maceration and alcoholic fermentation, and seed-associated phenolics are extracted in the latter part of maceration and alcoholic fermentation (Koyama et al., 2007b). Cold soaking arguably extends the early part of maceration, whereas extended maceration lengthens the latter part of the extraction phase. This suggests that cold soaking may offer greater extraction of skin-associated phenolics, and extended maceration greater extraction of seed-associated phenolics. The results reported here suggest that seed-associated phenolics may contribute to the faster

formation but transient stability of colour, whereas although skin-associated phenolics, although slower to polymerise, apparently provide more enduring wine colour.

The freeze-thaw treated wines at 30 months bottle age had a higher tannin concentration (3.7-fold higher) than control wines but had a similar non-bleachable pigment concentration to four of the five remaining treatments and no detectable anthocyanin, suggesting that the freeze-thaw treated wine may have lacked sufficient anthocyanin to capitalise on its high tannin concentration for the formation of additional non-bleachable pigment. The decline in non-bleachable pigment concentration observed in the extended maceration treatment wines from six to 30 months bottle age, and the reduction in hue of those wines suggests that while some of the non-bleachable pigment may not have been stable to 30 months bottle age, there may have been an overall increase in the quality of the non-bleachable pigment, which conferred more stable colour on the aged wine. Higher hue is indicative of ruby-garnet coloured wine, and infers either the early maturation of the wine due to the formation of pigment-flavanol polymers or the production of pyranoanthocyanin (vitisin) complexes (Cheynier et al., 2006). Lower hue values (blue-purple coloured wine) can have been related to greater anthocyanin stacking in co-pigmentation complexes, which have been associated with a bathochromic shift to absorbance of light of longer wavelengths in wines, but in the case of extended maceration wines, the anthocyanin concentrations were low, suggesting that the blue-purple colour may have been due to enhanced formation of portisins (Marquez et al., 2013).

3.5.2 Industry implications

In practice, the decision to use a particular maceration process in winemaking will be guided by evaluation of the costs (e.g. time, winery capacity, inputs) versus the benefits (e.g. greater phenolic concentration in wine, more balanced phenolic profile, product

consistency). Four of the six maceration treatments examined in this study conferred greater phenolic concentration on Pinot Noir wines but each treatment had cost implications. The application of enzyme treatment in winemaking has the lowest option in production cost and uses the least tank space (8 days). However, this treatment was shown to confer no phenolic advantage. The cold soak treatment used energy and cool store space but conferred greater non-bleachable pigment concentrations in aged wine. The freeze-thaw treatment required relatively high energy costs and freezer capacity producing a highly tannic wine. This option may be most appealing for winemakers seeking to use a small batch of highly tannic Pinot Noir wine in blending. The extended maceration process consumed fermentation tank space for 12 days, and the wines ran the risk of over-extraction (particularly of seed tannin) (Joscelyne, 2009), wine oxidation, and contamination by the aerobic, volatile acid-producing *Acetobacter* bacterium. While the energy and capital expense of microwave maceration was high, at 30 months the wines showed responses similar to wines from the cold-soak treatment. The microwave maceration treatment wines had finished fermentation after eight days skin contact time as did the control wines.

3.6 CONCLUSION

This study demonstrated that the choice of maceration technique is an important part of the Pinot Noir winemaker's tool-kit for manageing extraction and stabilisation of phenolics in this notoriously challenging variety. The occasionally maligned cold soak maceration treatment was demonstrated to have value for long term colour stability, which may justify the use of this process in the face of time and fermenter occupancy pressures during vintage. The freeze-thaw treatment results suggested that this method may be effective

for producing very tannic wines, which may offer winemakers a blending option during vintages where grape tannin is low or winery capacity constraints limit skin contact time during alcoholic fermentation. Further research is needed to understand why the extended maceration treatment wines declined in non-bleachable pigment concentration after 30 months bottle age. For example, whether the source of tannin (seed or skin) or tannin composition in the three highly extractive maceration treatments differed from each other, and if this difference contributed to the decline in non-bleachable pigment concentration observed here. For practical purposes, the decline in stable colour observed in extended maceration wines may offer no disadvantage given that those wines remained significantly higher in stable colour (52%) than control wines at 30 months bottle age. An important point is that the non-bleachable pigment and tannin concentration of microwave and extended maceration treatments were similar at 30 months bottle age. This suggests that microwave maceration approach may offer a time-saving alternative to extended maceration winemaking. However the consideration of the energy costs and practical application of this technique requires further investigation.

While the phenolic composition of the wine has a major influence on wine colour stability the analysis of aroma and flavour characteristics of would provide further insight into the quality of the wine in response to the maceration technique imposed.

Appendix 3.1 Pinot Noir wine phenolics at bottling, 50 days post-inoculation (mean; n=4) for six maceration treatments.

Phenolic character	control	enzyme	micro-wave	cold-soak	freeze-thaw	extended maceration	LSD (trt)
Total phenolics (AU)	22.1b	22.3b	30.3a	19.7b	29.7a	20.1b	3.65
Total pigment (AU)	7.88b	7.40c	13.1a	7.03c	9.59b	5.00d	1.50
Anthocyanin (mg/L)	147c	138c	247a	132c	182b	80.0d	28.8
NBP (AU)[Ŧ]	0.31c	0.31c	0.43b	0.27c	0.30c	0.60a	0.09
Tannin (g/L)	0.00b	0.01b	0.29a	0.00b	0.31a	0.00b	0.13
Colour density (AU)	2.23c	2.34c	3.72a	2.16c	2.58c	3.21b	0.44
Hue (AU)	0.69	0.69	0.66	0.68	0.66	0.69	0.03

Significant differences in concentration for each parameter ($P \leq 0.05$) are represented by different letters.

[Ŧ]NBP, Non-bleachable pigment.

3.7 SUBSEQUENT RESEARCH FOCUS

Having demonstrated that the maceration technique used during winemaking imparts considerable differences to the wine phenolic composition, subsequent experiments investigated the role of different berry tissues on the wine phenolics with the view to choosing a maceration technique that resulted in a preferred wine style. The following chapter describes the influence of individual berry tissues isolated from Pinot Noir grapes on the phenolic composition of Pinot Noir wines. In the first experiment the total tannin and anthocyanin content of the berry tissues extractable in 50% (v/v) ethanol was determined and in two further experiments wines were made in which the role of each berry tissue was amplified either by omitting it from the must or doubling its content in the must. In this way the contribution of each tissue to the phenolic profile of the wine was emphasized as the wine aged from 6 to 12 months.

4

THE CONTRIBUTION OF COMPONENT BERRY TISSUES TO THE PHENOLIC COMPOSITION OF PINOT NOIR WINE

Experiments forming parts of this chapter were presented as a poster and Pinot Noir workshop at 8thth International Cool Climate Symposium, Hobart, Australia, February 2012.

Title: 'Seeing Double Pinot Phenolics'

Authors: Angela M. Sparrow, Robert G Dambergs and Dugald C Close

The chapter is in preparation as a research article to be submitted to American Journal of Enology and Viticulture.

Title: 'The contribution of component berry tissues to the phenolic composition of Pinot Noir wine'

Authors: Angela M. Sparrow, Robert G Dambergs, Keren A Bindon, Paul A Smith and Dugald C Close

4.1 ABSTRACT

Berry tissue components (skin, seed and pulp) were isolated from Pinot Noir grapes to determine the tannin content of each component. In addition the role of each berry component in determining the phenolic profile of the wine was investigated by omitting or doubling each berry tissue in the must and fermenting these using submerged cap micro-vinification. Free anthocyanin, total tannin and non-bleachable pigment concentration in juice and wine were examined at seven time intervals from yeast inoculation (day 0) to 12 months bottle age (day 400). When included in the must, berry pulp was associated with reduced wine tannin concentration, an effect thatwas more selective for seed tannin. Consequently the tannin composition of wines made with whole-berry ferments most closely reflected that of skin tannin. The formation of non-bleachable pigments was greater in treatments where seeds were present during fermentation. However, when the contribution of seed tannin in wine was increased by doubling the complement of seeds in

the must, anthocyanin concentration remained constant and non-bleachable pigments did not increase during ageing. Treatments with a double complement of grape skins were found to enhance both non-bleachable pigment and tannin in aged wines.

4.2 INTRODUCTION

Polyphenolic wine tannins can be extracted from the skin, seed and stalk of the grape bunch, and colour pigments (anthocyanins) are extracted from grape skins (Souquet et al., 2000, Jordao et al., 2001, Bindon et al., 2010a, Ribereau-Gayon et al., 2006). During winemaking, non-bleachable pigments are formed by polymerisation of anthocyanins with tannins (Pastor del Rio and Kennedy, 2006). The polymeric tannin-pigment complexes are more stable than free anthocyanins at wine pH and are resistant to sulphur dioxide bleaching (Somers, 1971). In addition, a proportion of non-bleachable wine colour is derived directly from reaction products of anthocyanin (Somers, 1971, Boulton, 2001, Cheynier et al., 2006). By comparison with other red grape varieties *Vitis vinifera* cv. Pinot Noir grapes have reduced colour development and a low skin to seed tannin ratio, factors which influence both the final tannin profile and colour stability of the wine. However the relative importance of grape-derived tannins from each berry component involved in the development of wine colour and the formation of non-bleachable pigments is poorly understood.

While colour stability may be enhanced by the addition of oenological tannins (Bautista-Ortín et al., 2005, Neves et al., 2010, Versari et al., 2013, Gao et al., 1997, Romero and Bakker, 2001), the selection of commercial oenological tannins requires caution, as in some instances the desired effects of improved wine colour and colour stability can be reversed

because of the preparation and consequent composition of the oenological tannins (Bautista-Ortín et al., 2005, Bautista-Ortin et al., 2007, He et al., 2012a).

In studies of red wine grapes, tannins derived from different parts of the berry have been characterised by their molecular mass and subunit composition, and these properties have been found to influence aspects of wine character such as flavour, mouth-feel and colour stability of the wine (Somers, 1971, Vidal et al., 2004b, Ducasse et al., 2010a, Mercurio et al., 2010, McRae and Kennedy, 2011). The presence of seeds in the must of Pinot Noir ferments compensates for the low concentrations of tannin in the skin and these seed-derived tannins are likely to have a central role in colour stability and mouth feel of the wine. The relative contribution of anatomically distinct berry tissues (skin, seed, pulp) to wine quality has been difficult to differentiate because of the disruption of the component tissues caused by de-stemming and crushing prior to vinification. By contrast, the phenolic composition of wine grapes has been assessed using a range of different extraction techniques and solvents (Saint-Criq et al., 1998, Cayla et al., 2002, Iland et al., 2004, Sarneckis et al., 2006) however, studies have shown that less than 50% of the grape tannin becomes incorporated into the wine (Harbertson et al., 2002, Adams and Scholz, 2008). In order to more accurately predict the proportion of tannin extracted from grapes to wine, grape tannin extraction during fermentation have been explored using a more wine-like medium, for example consisting of 12% aqueous ethanol, sulphur dioxide and tartaric acid, adjusted to pH 3.2, (Mattivi et al., 2009). However, Scollary (2010) cautioned that a wine-like medium did not account for changing sugar concentration and temperature variability that occurs during fermentation.

Differences in skin and seed tannin extractability, and the subsequent selectivity of tannin adsorption by cell wall material of Pinot Noir are unknown, but potentially contribute to

the loss of extractable tannin in the wine. Maceration techniques such as cold soaking, extended maceration and thermovinification are often employed to improve the extraction of both pigment and tannin in red wine (Sacchi et al., 2005) but the tissue source of the tannin is not necessarily discriminated.

This study aimed to address these issue using two new protocols: the first differentiated the source of grape polyphenols by isolating Pinot Noir berry tissue components (skin, seed and pulp) and extracting them in 50% (v/v) ethanol; the second compared the phenolic composition of wines made from berry tissue components either in isolation or in combination with other berry tissue components. Each of the reconstructed 'musts' used in the second protocol contained freshly pressed Pinot Noir grape juice which provided a medium in which the alcohol concentration gradually increased from 0-12.0% during fermentation, exposing the grape polyphenolic compounds to typical changes that occur during fermentation such as declining sugar concentration, development of yeast metabolites, and the change of organic grape acids (tartaric and malic) to include acetic, butyric and succinic acids found in wine (Mato et al., 2007).

Subsequently, changes in the concentration of total tannin and anthocyanin of the micro-vinified wines, together with the development of non-bleachable pigments were assessed over a 12 month period of wine ageing.

4.3 MATERIALS AND METHODS

4.3.1 Grape sampling and replication

Grapes of *Vitis vinifera* cv. Pinot Noir G5V15, from drip irrigated vines, trained to vertical shoot position, were hand harvested from a vineyard in northern Tasmania in April of 2011. Whole bunches of grapes at 21.7 ^{o}Brix were randomly allocated to four 10 kg replicates.

From each replicate, 100 berries were selected at random to determine berry mass and fruit composition. Grape juice total soluble solids (°Brix) were measured using a hand-held refractometer, pH was measured with a Metrohm pH meter/autotitrator, and titratable acidity by titration with 0.333 M NaOH to an end point of pH 8.2 (reported as g/L tartaric acid). Two hundred grams of berries from each replicate were frozen at -20 ^{o}C for later analysis of grape colour and tannin (Iland et al., 2004, Sarneckis et al., 2006, Dambergs et al., 2012b).

4.3.2 Extraction of berry components

Grapes from each of the four 10 kg replicates were randomly hand-plucked to prepare four 50 g berry samples for extraction. Grape component separation was effected by expelling the pulp and seeds from individual berries to obtain the skins. Forceps were then used to isolate the seeds from the pulp. The pulp and seeds were expelled from a further sample of grapes to determine the tannin concentration of the pulp plus seeds. The tannin concentration of the berry and isolated components was determined by extracting the phenolic compounds using the method described by Sarneckis et al. (2006) followed by rapid tannin analysis (Dambergs et al., 2012b). It was assumed that each of the berry tissues had equivalent extractability in 50% (v/v) ethanol. In order to thoroughly homogenise the isolated berry components for extraction, each 50 g sample was combined with 100 mL of an isotonic sucrose buffer consisting of 24% sucrose and 8 g/L tartaric acid adjusted to pH 3.3 with 5 M sodium hydroxide to a final homogenate mass of 150 g. Each berry component sample was homogenised at 8000 rpm for 20 seconds in a Retsch Grindomix GM200 homogeniser with an S25 N-18G dispersing element (Janke & Kunkel GmbH & Co, Germany) fixed with a floating lid. A 1 g portion of whole berry or berry component homogenate was subsequently suspended in 10 mL of 50% v/v ethanol/water,

pH 2. Samples were extracted with shaking at room temperature for 1 h, and then centrifuged for 15 min at 5000 rpm. Extracts were analysed for total tannin as described below, accounting for the dilution in sucrose buffer for each sample.

4.3.3 Preparation of berry components for vinification

The micro-vinification technique used in this study allowed us to ferment four replicates each of 200 g from each berry component (whole berries, skin, seed, pulp, juice).

For the control microvinification treatment 200 g of berries were removed from the stalks by hand from each of the four 10 kg bunch replicates described previously. A further 200 g of berries from each replicate was dissected as described for the tannin extraction protocol above, to isolate a full complement of each grape tissue component from 200 g of grapes. The process was repeated with two further 200 g samples of berries to prepare treatments for the evaluation of potentially interactive effects between the grape components. Treatments prepared were (1) berries (control); (2) seeds + pulp; (3) skins + pulp; (4) seeds + skins; (5) skins; (6) seeds; (7) pulp; and (8) juice. The juice treatment was included specifically to be used as a blank to counteract any background absorbance during UV-VIS spectral analysis. The grape juice was expressed from a 6 kg sample of grapes originating from the same source as the grapes used to prepare the experimental treatments. Juice from the crushed grape bunches was filtered through 2 x 2 mm stainless steel mesh to obtain fresh juice. The juice was added to the berry component treatments to adjust the mass of each 'must' to 200 g then placed in 250 mL polycarbonate containers fitted with a screw-cap.

4.3.4 Preparation for vinification of whole berries with a double complement of skin, seed or pulp components.

A second microvinification experiment was conducted to further evaluate the effect of each berry tissue component in the wine by doubling the contribution of that component in whole berry fermentations. To prepare these treatments, berries from each of the four 10 kg fruit replicates described above were removed from the stalks by hand and divided into five 200 g groups. One group formed the whole berry control, a second 200 g group was dissected into skins, seeds and pulp components with one berry component then added to each of three remaining groups of 200 g berries. This effectively doubled the contribution of one berry component in each wine. Treatments for this trial were: (1) berries (control); (2) whole berries + skins; (3) whole berries + seeds; (4) whole berries + pulp.

4.3.5 Microvinification protocol

Prior to inoculation grapes were crushed in their respective fermentation vessels using a flat-ended wooden pestle. To each fermentation vessel 50 mg/L SO_2 was added as potassium metabisulfite and the must refrigerated overnight at 4^{o}C. The following day, musts were warmed to 25^{o}C and inoculated with 300 mg/L Lallemand RC212 yeast solution. The fermentation vessels were fitted with a fibreglass gauze sieve (115 mm diameter, mesh size 1 x 2mm) and a cylindrical PVC spacer (52 x 32mm) to effect submerged cap vinification (Dambergs and Sparrow, 2011, Smart et al., 2012). During fermentation the screw cap lid was loosely fitted to the vessel to provide adequate gas exchange and ferments incubated at 25°C (±1°C). On day 3 of the fermentation, 300 mg/L of diammonium phosphate was added to each ferment. Must samples of 1.5 mL (< 1% of total volume) were taken on alternate days during fermentation, and were frozen at -20 °C

for later phenolic analysis. The conclusion of fermentation was tested after six days using Clinitest® reagent tablets (Bayer Australia Ltd.) when each ferment was confirmed to contain less than 2 g/L of residual sugar. The must was pressed by inserting a second PVC spacer into the fermentation vessel with the gauze sieve facilitating separation of wine and solids. Wine was decanted from the fermentation vessel under CO_2 cover into amber glass bottles and sealed with polypropylene screw caps. Wines were stored at 4°C for 14 days, racked under CO_2 cover to 50 mL amber glass bottles and 80 mg/L SO_2 added. Following 30 days storage at 12°C the wine was racked under CO_2 cover into two 25 mL amber glass bottles and sealed with screw caps for storage at 12°C. For each treatment, 1.5 mL wine samples were taken at pressing, racking, bottling, 6 months and 12 months post-bottling. All samples apart from the aged wine samples were frozen at -20 °C prior to analysis.

4.3.6 Analysis of extracts and wine

Frozen samples were thawed at room temperature and clarified by centrifugation at 5000 rpm for 5 mins using an Eppendorf 5417C centrifuge with an Eppendorf F45-30-11 rotor (Crown Scientific, Australia). Extracts and wines were diluted with 1 M HCl and spectral analysis was performed using a UV-VIS Spectrophotometer (Model Genesys™ 10S Thermo Fisher Scientific Inc., Madison, WI, USA) scanning at 2 nm intervals for wavelengths 200 to 600 nm. Total tannin concentration was determined using the method described by Dambergs et al. (2012b). All samples were analysed for wine colour using a modification of the Somers assay described by Mercurio et al. (2007). Model wine buffer for colour analysis was prepared using 5 g/L potassium hydrogen tartrate in 12% (v/v) ethanol. Assays were performed following a 1:10 dilution of wine in either model wine buffer containing 0.1% (v/v) acetaldehyde or model wine buffer containing 0.375% (w/v) sodium metabisulfite.

In addition to the analyses described above, a subset of wine samples at 12 months bottle age were analysed for tannin composition. These analyses were conducted externally by AWRI (Australian Wine Research Institute) based in Glen Osmond, South Australia. Wine tannin concentration was determined using the methyl cellulose precipitable tannin assay (MCPT) according to Mercurio et al. (2007). MCP tannin assays gave the tannin concentration of each wine in epicatechin equivalents (mg/L).

Isolation of Total Polymeric Phenols: Solid-phase extraction (SPE) was used to isolate total polymeric phenols form each wine sample as described by Kassara and Kennedy (2011). In brief Oasis HLB cartridges (3 mL, 650 mg, 30 µm) were conditioned with methanol (2 mL) followed by water (2 mL). Wine (1 mL) was applied to the cartridge under gravity. Once wine phenolics were adsorbed, the cartridge was dried with a gentle stream of nitrogen. Each cartridge was washed with 20 mL of 95% acetonitrile: 5% 0.01 M HCl (v/v) under vacuum. This step removed phenolic acids, nonpolymeric flavanols, flavonols, anthocyanins and other pigmented monomers. Polymeric phenolics were eluted with 300 µL of near formic acid followed by 3 mL of 95% (v/v) methanol. The fraction was concentrated under nitrogen at 28 ^{o}C and dissolved in methanol (10g/L) based on the initial wine tannin concentration by MCPT.

Composition of Tannins Isolated from Wine: Tannins obtained from wines were characterized by acid catalysis in the presence of excess phloroglucinol (phloroglucinolysis) and subsequent HPLC analysis to determine the mean degree of polymerization (mDP) and subunit composition (Kennedy and Jones, 2001, Kennedy and Taylor, 2003a). Tannins isolated from wine using SPE were dissolved in methanol to 10g/L. An aliquot of this solution (25 µL) was reacted with 25 µL phloroglucinol reagent (0.2 N HCl, 100g/L phloroglucinol, and 20 g/L ascorbic acid in methanol) in a 0.2 mL PCR tube. The reaction

was heated at 50 ^{o}C for 25 min, cooled on ice for 1 min, and then quenched with 70 mM (150 µL) sodium acetate. Controls were prepared by replacing phloroglucinol reagent with methanol, (-) epicatechin was the HPLC standard, and calculations were as described by Kennedy and Jones (2001). HPLC analysis was performed using a model 1100 HPLC (Agilent Technologies Australia Pty Ltd., Melbourne, Australia) equipped with Chemstation software. The concentration of isolated polymer phenolics by phloroglucinolysis was found to be correlated (r^2 = 0.84) to the concentration of isolated polymeric phenolics when assessed using the MCPT assay.

Gel Permeation Chromatography (GPC): The GPC method was adapted from that described by Kennedy and Taylor (2003a) to allow for increased size distribution resolution of high molecular mass proanthocyanidins isolates which had been diluted 1:5 in dimethylformamide as described by Bindon and Kennedy (2011). The column arrangement and chromatographic conditions were the same as the original method. For calibration, a second order polynomial was fitted with the proanthocyanidin elution time at 50% for each standard. Pre-veraison skin fractions of known mDP were used as standards for calibration, and a second-order polynomial fit with the time at 50% elution for each standard. For GPC analysis, proanthocyanidin samples in methanol were diluted with 4 volumes of the HPLC mobile phase. The maximum amount of proanthocyanidin injected onto the column was 40 µg. The calibration was used to predict mean molecular mass, as described previously (Bindon et al., 2010a, Bindon et al., 2010b).

4.3.7 Statistical analysis

Statistical analyses were conducted using GenStat Release 13.1 Copyright 2010, VSN International Ltd. The mean and standard deviation for fruit composition characters and the phenolic content of grapes were calculated using one–way ANOVA. The mean

concentration of individual phenolic components in must and wine samples for each treatment replicate over the seven sampling periods (n=312) was compared between treatments using Repeated Measures ANOVA. Post-hoc analyses were determined using Fisher's Protected Least Significant Difference (LSD) test.

4.4 RESULTS

4.4.1 Grape composition

The fruit composition and phenolic content of Pinot Noir grapes used in this study are provided in Table 4.1. While the grape sample had slightly lower °Brix and pH; higher titratable acidity, and a large berry mass than other published work using this variety, these parameters remained within the acceptable range of commercial ripeness for this Pinot Noir. The tannin and anthocyanin concentration for the grape samples were within the range reported in literature for Pinot Noir (Cortell et al., 2005, Cortell et al., 2007, Cortell and Kennedy, 2006, Kemp et al., 2011).

The distribution of tannin within the grape sample was calculated from the tannin concentration of whole berries and the isolated berry components (Table 4.2). The tannin concentration of berries extracted in the presence of sucrose buffer was similar to that extracted using the method described by Sarneckis et al. (2006) (Table 4.1).

Table 4.1 Fruit composition (Mean and Standard Deviation, n=4) of fruit used in trials.

Fruit composition parameter		
Berry weight (g FW‡)	1.30	± 0.01
Total Soluble Solids (°Brix juice)	21.70	± 0.50
Juice pH	3.34	± 0.02
Titratable acidity (g/L juice)	9.16	± 0.22
Anthocyanin (mg/g berry FW‡)	0.40	± 0.01
Total tannin (mg/g berry FW‡)	4.88	± 0.21

‡FW, fresh weight

The highest concentration of tannin in Pinot Noir grapes was found in the seeds. Tannin content per berry and tannin concentration per unit fresh weight of the components analysed were found to be 22 times higher in seeds than in the skins. As the skin constituted 29% of the berry mass and the seeds 3.8%, this represented a skin to seed tannin ratio of 1:2.9 in the berries (Table 4.2) which is similar to other reports for Pinot Noir (Cortell et al., 2005, Cortell et al., 2007, Cortell and Kennedy, 2006, Kemp et al., 2011).

Table 4.2 Distribution of tannin in grape berry components and their contribution to total fresh weight (FW) (Data are the mean ± standard deviation, n=4)

Berry component	Component[Ƀ] (%) (FW[‡]/berry)	Tannin[§] (mg/g) (FW[‡] of component)	Tannin[†] (mg/berry)
whole berry	100	4.6 ± 0.1	5.5 ± 0.2
juice	9.7 ± 0.6	nd	nd
skin	29 ± 2.6	4.3 ± 0.1	1.6 ± 0.1
seed	3.8 ± 0.3	95 ± 1.3	4.7 ± 0.4
pulp[Ŧ]	53.5 ± 1.7	0.7 ± 0.1	0.5 ± 0.1

[‡]FW, fresh weight; [Ƀ]Average mass of each berry component recovered per 100 g of berries; [§]Determined per unit fresh mass of juice, skin, seed and pulp recovered; [†]Tannin concentration per component expressed per berry; [Ŧ] Calculated by difference, (pulp + seed)-(seed); *nd*, not detected.

4.4.2 The effect of skin, seed and pulp on wine tannin concentration

Tannin concentration was determined on alternate days from inoculation to the end of ferment (day 6), then at racking (day 20), bottling (day 50), 6 months (day 230) and 12 months (Table 3). The tannin concentration of the 'whole berry' control treatment gradually increased by 38% from day 2 to day 6 of fermentation and then remained steady at this concentration to 12 months bottle age. In terms of net grape extraction, the control represented a recovery of 10% in the wine (Table 4.3).

Table 4.3 Tannin concentration (g/L) during fermentation and a 12 month ageing period in Pinot Noir wines made from berry components

	Days post inoculation							
Berry component	0	2	4	6	20	50	230	400
‡	Tannin concentration (g/L)							
whole berries	nd	0.03 c	0.30 c	0.42 c	0.45 b	0.42 c	0.49 c	0.54 b
seed + pulp	nd	0.01 c	0.02 d	0.05 d	0.05 d	0.04 d	0.04 d	nd
skin + pulp	0.09 b	0.44 b	0.56 b	0.52 bc	0.53 b	0.55 bc	0.55 bc	0.40 b
seed + skin	0.13 a	0.66 a	1.17 a	1.38 a	1.41 a	1.50 a	1.56 a	1.49 a
skin	0.08 b	0.55 ab	0.64 b	0.63 b	0.59 b	0.64 b	0.64 b	0.49 b
seed	0.01 b	0.06 c	0.26 c	0.46 c	0.42 b	0.48 c	0.50 c	0.35 b

For each trial, numbers within a column followed by different letters indicate significant differences at $P \leq 0.05$. ‡Tannin concentration monitored during fermentation and ageing using the predictive tannin assay: Repeated measures ANOVA (treatment x time) F= 69.23 df 42, 147 P <0.001; LSD 0.05 = 0.15; *nd*, not detected.

By contrast, the treatment containing 'skin + seed' in the absence of pulp material had a significantly higher tannin concentration than the control from the early stages of fermentation through to 12 months bottle age, indicating that the presence of pulp material reduced wine-extractable tannin by a factor of 3.

The relative contributions of grape skin and seed to wine tannin demonstrated that tannin extracted from skins exceeded seed tannin extraction during fermentation, although these differences were less evident after 6 and 12 months of ageing.

When a second complement of seeds was added to whole berry must, effectively doubling the seed contribution to the wine, the tannin concentration increased 4-fold relative to the control treatment by day 6 of fermentation and remained at this concentration through to 12 months bottle age when it was 2-fold higher than the control (Table 4.4).

The inclusion of a second complement of skins in the whole berry ferment increased the tannin concentration 2-fold by day 2 of fermentation and it continued to increase in parallel to the control such that at 12 months bottle age it too, was 2 –fold higher than the control wines (Table 4.4). The inclusion of a second complement of pulp components had a distinctively different effect reducing the tannin concentration of the wine from day 6 of fermentation through to six months bottle age, by which time no tannin was detected. Comparing the results of the impact of pulp tissues on the contribution of seed and skin tannin (Table 4.3) suggests that tannin was extracted more readily from the seed when pulp was absent and that the amount of seed tannin retained in the ageing wine was reduced by the presence of pulp derivatives. Skin tannins did not appear to be affected to the same extent.

Table 4.4 Tannin concentration (g/L) from inoculation to 12 months bottle age for Pinot Noir wines with a double complement of skin, seed or pulp berry components.

	Days post inoculation							
Berry component treatment	0	2	4	6	20	50	230	400
	Tannin concentration (g/L)[‡]							
whole berries	nd	0.34 b	0.41 c	0.47 c	0.48 b	0.52 c	0.57 c	0.76 b
whole berries + skin	nd	0.72 b	0.70 b	0.76 b	0.72 b	0.76 b	0.79 b	0.95 a
whole berries + seed	nd	0.68 a	1.43 a	1.86 a	1.86 a	1.86 a	1.86 a	1.86 a
whole berries + pulp	nd	0.12 b	nd	0.28 c	0.10 d	0.14 d	nd	0.27 c

For each treatment, numbers within a column followed by different letters indicate significant differences at $P \leq 0.05$. [‡]Tannin concentration monitored during fermentation and ageing; repeated measures ANOVA (treatment x time) F= 25.6 df 21, 84 P = 0.02; LSD 0.05 = 0.27; nd, not detected.

4.4.3 Extraction of anthocyanin and the development of non-bleachable pigments

For each of the experiments outlined, the concentration (mg/L) of free anthocyanin and the development of non-bleachable pigments (bisulphite resistant) were also determined in the wines that had been exposed to skin contact (Table 4.5).

The conversion of anthocyanin to non-bleachable pigments was lowest in treatments where seeds were absent during fermentation. This effect was further enhanced during the wine ageing process, and resulted in a reduced conversion of anthocyanin in wines which had not been exposed to seeds. Consequently, by 12 months bottle age, treatments that contained only skins during fermentation, i.e. 'skin' and 'skin + pulp' treatments were significantly lower (46%) in non-bleachable pigment concentration than the 'whole berries'

or 'skin + seed' treatments. This result suggests that the presence of seeds during ferment significantly contributed to the formation of non-bleachable pigment.

Table 4.5 Anthocyanin concentration (mg/L) and non-bleachable pigment development during fermentation and a 12 month ageing period in Pinot Noir wines made from berry components

Berry component	Days post inoculation							
treatment	0	2	4	6	20	50	230	400
	Anthocyanin (mg/L)[‡]							
whole berries	62.4 a	130 ab	157 a	163 a	140 a	135 a	95.6 ab	58.6 b
skin + pulp	37.4 b	126 b	156 a	160 a	137 a	140 a	111 a	76.7 a
seed + skin	44.7 b	145 a	158 a	153 a	135 a	133 a	87.6 b	45.9 b
skin	27.0 b	155 a	161 a	169 a	138 a	147 a	119 a	79.9 a
	Non-bleachable pigment (AU)[§]							
whole berries	0.12	0.23 a	0.26 a	0.26 a	0.28 a	0.30 a	0.57 a	0.98 a
skin + pulp	0.03	0.17 a	0.18 b	0.16 b	0.18 b	0.18 b	0.18 c	0.58 b
seed + skin	0.04	0.19 a	0.24 a	0.24 a	0.27 a	0.28 a	0.29 b	1.08 a
skin	0.03	0.17 a	0.18 b	0.15 b	0.17 b	0.18 b	0.17 c	0.52 b

For each trial, numbers within a column followed by different letters indicate significant differences at $P \leq 0.05$. [‡]Anthocyanin concentration monitored during fermentation and ageing, treatments containing only seeds are excluded. Repeated measures ANOVA, (treatment x time) F=35.8 df 42,147 $P<0.001$; LSD 0.05 = 16.4; [§]Non-bleachable pigment assessed as absorbance units, treatments containing only seed excluded: Repeated measures ANOVA, (treatment x time) F=16.36 df 42,147 $P<0.001$; LSD 0.05 = 0.13.

Treatments that had a double complement of skins (whole berry + skins) were significantly higher in free anthocyanin than the whole berry control from day 2 of fermentation through to bottling, 50 days after inoculation (Table 4.6). In both of these treatments the anthocyanin concentration decreased 3-fold from bottling to 12 months bottle age and a concomitant increase in the development of non-bleachable pigment was observed. When the seed complement was doubled, the highest anthocyanin concentration was detected at day 4 of fermentation at which time it was similar to the 'whole berry' control treatment. However by 12 months bottle age, the anthocyanin concentration of the control treatment had declined by 3-fold, while the anthocyanin concentration of wines with a double complement of seeds was unaltered. Unlike the previous treatments, the development of non-bleachable pigment did not follow a complementary pattern. Rather, the relatively small concentration of non-bleachable pigment that developed during fermentation was apparently compromised, and declined by 34% between bottling and 12 months of wine ageing resulting in a concentration that was 68% less than that of the control wines.

Table 4.6. Anthocyanin concentration (mg/L) and non-bleachable pigment development during fermentation and a 12 month aging period in whole-berry Pinot Noir wines to which skin, seed and pulp berry components were added.

Berry component treatment	Days post inoculation							
	0	2	4	6	20	50	230	400
	Anthocyanin concentration (mg/L) ‡							
whole berries	21.7 a	129 c	145 b	134 c	122 c	139 b	95 b	47.0 b
whole berries + skin	29.2 a	272 a	261 a	248 a	232 a	244.a	173 a	86.4 b
whole berries + seed	24.2 a	144 bc	165 b	165 bc	165 b	158 b	165 a	165 a
whole berries + pulp	15.0 a	178 b	158 b	203 b	152 bc	161 b	105 b	81.2 b
	Non-bleachable pigment (AU) §							
whole berries	0.12 a	0.25 a	0.28 a	0.24 a	0.27 a	0.30 a	0.59 b	1.08 b
whole berries + skin	0.22 a	0.40 a	0.38 a	0.35 a	0.38 a	0.45 a	0.81 a	1.85 a
whole berries + seed	0.11 a	0.29 a	0.37 a	0.35 a	0.35 a	0.47 a	0.35 c	0.35 d
whole berries + pulp	0.16 a	0.24 a	0.27 a	0.26 a	0.23 a	0.28 a	0.37 c	0.67 c

For each trial, numbers within a column followed by different letters indicate significant differences at P ≤ 0.05. ‡Anthocyanin concentration during fermentation and ageing analyzed by repeated measures ANOVA, (treatment x time) F=17.8 df 21,84 P<.001; LSD 0.05 = 40.8; §Non-bleachable pigment assessed as absorbance units where treatments containing only seed were omitted: Repeated measures ANOVA, (treatment x time) F=15.95 df 21,84 P <.001; LSD 0.05 = 0.21.

In treatments where the pulp complement was doubled (whole berry + pulp) there was no observable difference in the anthocyanin concentration of the wine relative to the 'whole berry' control whereas the concentration of non-bleachable pigment declined by 38% between bottling and 12 months bottle age, once again suggesting that a proportion of the pigments formed earlier may have become unstable (Table 4.6). A possible explanation is that the non-bleachable pigments formed when anthocyanins and seed tannins were bound together during fermentation, were more susceptible to structural breakdown as the wine aged due to the simpler long-chain structure of the pigmented polymers (Figure 4.1 and Table 4.7).

4.4.4 Tannin subunit composition of berry tissue wines

The results were interpreted further using tannin compositional analysis on a sub-set of the experimental wines at 12 months bottle age. The analyses, conducted in duplicate, confirmed the results for tannin determination by spectral analysis with seed + skin based wines having a higher concentration of tannin that the whole berry control wines (Table 4.7).

No tannin was detected in wines made from juice or pulp tissue. MCPT analysis also showed that when 'seeds + pulp' components were fermented together, no tannin was detected in 12 month old wines despite the observation that wine made from isolated seed components had a tannin concentration of 0.45 g/L. The pulp components apparently had less impact on tannin concentration for wines made from 'skin + pulp' ferments as these had only 16% less tannin than the wines made from 'skin' components alone. Wines made from 'seed' components, were found to contain no trihydroxylated tannin subunits but had 4-fold higher percentage of galloylated tannin subunits than wines made from isolated skin components (Table 4.7).

Table 4.7 Tannin compositional analysis of wine made berry components of Pinot Noir at 12 months bottle age

Treatment	MCPT[†]	mDP[‡]	% MC[§]	% tri-OH[₸]	% gall[€]	tri-OH: gall[ϖ:]	50% GPC[¥]
whole berries	0.67	5.5	34.8	16.7	3.8	4.4	1866
skin + pulp	0.27	6.7	38.7	22.3	2.7	8.3	1545
seeds + pulp	nd	nd	nd	nd	nd	nd	nd
skins + seed	1.19	4.2	50.6	8.00	6.2	1.3	2057
skins only	0.33	8.1	61.0	25.5	2.3	11.0	1585
seeds only	0.45	2.1	56.3	nd	9.3	nd	1704

[‡]Mean degree of polymerization (mDP) based on subunit composition from phloroglucinolysis; [§]Mass conversion = % recovery of tannin subunits by phloroglucinolysis based on the tannin concentration (in g/L) determined by [†]methyl cellulose precipitation; [₸]% trihydroxylated tannin subunits; [€]% galloylated tannin subunits; [ϖ]ratio of trihydroxylated tannin subunits to galloylated tannin subunits; [g]molecular size at 50% GPC (g/mol); *nd*, not detected.

The mDP of wines made from 'skin' components alone was 4-fold higher than wines made from 'seed' components alone, while tannin molecular mass (or hydrodynamic volume) determined at 50% elution by GPC was 8% less in skin-based wines than in seed-based wines. Wines made from 'skin + seed' components in the absence of pulp, had lower mDP but higher molecular size than the wines containing a full complement of berry components. These results indicate that Pinot Noir seed tannins may be less compact but with longer chain length than skin tannins and consequently more susceptible to binding by pulp components during vinification. A diagram based on our observations proposing the physical and chemical mechanisms behind the influence of berry components during Pinot Noir winemaking is presented in Figure 4.1.

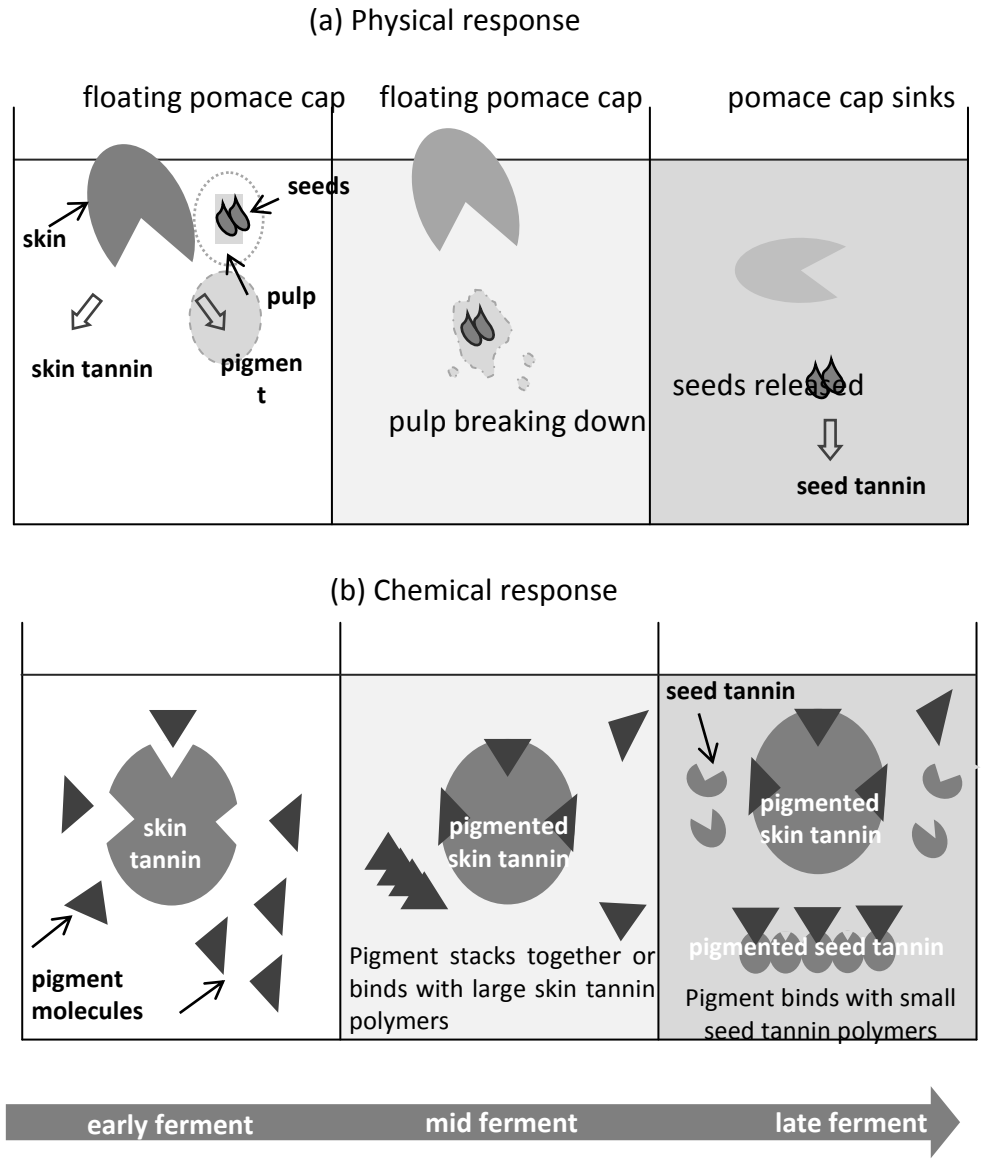

Figure 4.1 Proposed formation of pigmented tannin

(a) physical and (b) chemical responses during grape fermentation. Early in fermentation anthocyanin and tannin are released from the skins of the floating pomace. Mid-way through fermentation the pomace has lost colour, the pulp breaks down and free anthocyanin becomes co-pigmented or polymerises with large skin tannin polymers. Late in fermentation the seeds are released from the pulp, the floating pomace cap sinks and free anthocyanin polymerises with smaller seed tannin polymers.

4.5 DISCUSSION

This study demonstrated several important interactions between grape berry components and the extraction and stabilisation of phenolic compounds in Pinot Noir wine. Grape pulp had greater impact on the concentration of seed tannins than of skin tannins during vinification; anthocyanin extraction increased in the presence of both skin and seed tannin; and non-bleachable pigments formed from polymerisation of pigments with seed-derived tannins were not retained in wines after 12 months of bottle ageing.

From previous studies covering a range of Pinot Noir cultivars, it is widely accepted that Pinot Noir wine tannins come mainly from the grape seed which may contribute significantly to total wine tannin (Hardie et al., 1996, Hayasaka et al., 2003, Peyrot des Gachons and Kennedy, 2003, Kennedy, 2008, Mattivi et al., 2009), and this study reports a skin to seed tannin ratio of 1:2.9 in the grapes and a tannin recovery rate from grapes to wine of 10% which falls between the rates (4 to 16%) reported by Harbertson et al. (2002). In terms of viticultural effects, variability in Pinot Noir grape skin tannin concentration appears to more strongly relate to wine tannin concentration than does seed tannin concentration (Cortell et al., 2005) and the results of this study indicate why that might be so.

A clear interaction between grape pulp components and seed tannins was observed both during extraction of grape phenolic compounds in 50% (v/v) ethanol and during vinification. When combined grape and seed components were extracted in 50% (v/v) ethanol a significant amount of tannin was detected in the pulp, however in wines made from pulp components alone, no tannin was detected either by spectral analysis or methyl cellulose precipitation. When wine was made from must composed of ‘seed + pulp’ components, the seed tannin concentration of the wine was less than that made with seed components

alone, whereas skin-derived tannins were not compromised by pulp components in the same way. The tannins derived from seeds of several red wine grape varieties have been differentiated from skin tannins on the basis of their lower molecular mass and higher level of galloylation (Vivas et al., 2004, Bindon et al., 2010b), however working with Pinot Noir grapes we found that the seed tannins appeared to have higher molecular mass than skin tannins, yet had lower mDP than the skin tannins.

As seed tannins are recognised as having fewer cross-linkages (Bindon et al., 2010b), they may form long-chain polymers rather than more compact polymers, (Bindon, personal communication 2012). Indeed long-chain tannin polymers may form that have higher molecular mass than more compact tannin polymers, but these polymers would be more susceptible to depolymerisation by phloroglucinolysis resulting in a higher mass conversion ratio and lower mDP than skin tannins, as found in the tannin compositional analysis in this study.

Research has also shown that some of the tannins extracted during fermentation bind with polysaccharides from the cell walls of the skin and grape pulp, and are subsequently removed from the fermenting wine matrix with the grape marc (Hanlin et al., 2010, Bindon et al., 2010a). In support of this observation, this study demonstrated that doubling the grape pulp components in the must resulted in a significant reduction in the tannin concentration of wines at 12 months bottle age. Comparison of the time course for wine made with 'skins +pulp' to that of the wine made with 'seeds + pulp' suggested that the molecular interaction of the grape pulp components with tannins from the two berry components differed, and this interaction had a significant impact on both the composition and concentration of tannin detectable in the wine. No tannin was detected in wine made with 'seeds + pulp' although in wines made with 'seeds only' a considerable amount of

tannin was detected. The data suggest that seed tannins are selectively adsorbed by pulp during fermentation, and as a result, wines made from whole Pinot Noir grapes are compositionally indicative of skin tannin, as specified by a relatively higher mDP, high percentage of trihydroxylated subunits and lower percentage of galloylated subunits. In addition, wine made in the absence of pulp components was 3-fold higher in tannin concentration than the control wine, and had higher mDP and % MC, and a lower ratio of trihydroxylated to gallolyated subunits. These findings support the hypothesis that tannins, particularly seed tannins, interact with pulp components and are effectively removed from the wine matrix, although constraints on tannin extraction caused by pulp components cannot be ruled out.

The use of grape cell-wall fibres as a fining agent has been recently reported (Bindon and Smith, 2013a, Guerrero et al., 2013). These researchers found that cell wall-tannin interactions caused the removal of soluble tannin from wine during the fermentation process through polysaccharide-tannin binding. In their review on condensed tannin and grape cell wall interactions Hanlin et al. (2010) reported that the tannins of higher molecular mass are more likely to become bound to cell wall polysaccharides than tannins of lower molecular mass. Guerrero et al. (2013) found that when fibres from grape pulp cell wall material were added to wine there was a reduced affinity for tannins rich in epi-gallocatechin (skin tannins) and a high affinity for terminal epicatehin-3-*O*-gallate (seed tannins). The current study found that the Pinot Noir seed tannins (9.3% galloylation) were not only present in higher concentrations but had higher molecular mass and percentage galloylation than the skin tannins (2.3%) and may have become preferentially bound by pulp polysaccharides.

Not surprisingly wines made with a double complement of skins had significantly higher anthocyanin concentrations than the remaining treatments through to 6 months bottle age. However by 12 months bottle age the wines of each treatment had responded quite differently. While anthocyanin concentrations declined for the majority of the treatments, corresponding to a simultaneous increase in non-bleachable pigment development, the wine made with a double complement of seed was the exception. The free anthocyanin concentration of wine at 12 months bottle age that had been made with 'whole berries + seed' was 3.5-fold higher than the control treatment. Such a response of seed addition to red wines was also reported by Canals et al. (2008) who noted a concomitant decrease in anthocyanin concentration when seeds were removed. As no anthocyanin is contributed by additional seeds, the high free anthocyanin value observed must be attributed either to the anti-oxidant properties of phenolic compounds from the seeds (Plumb et al., 1998, Makris et al., 2007) causing some degree of stabilisation of the free anthocyanins in the wine matrix, or to the enhanced extraction of anthocyanin in the presence of seed components. Reviewing the role of the copigmentation of anthocyanins Boulton (2001) highlighted that free anthocyanins are generally more susceptible to oxidation than are copigmented anthocyanins or pigmented polymers such that the concentration of free anthocynanins to pigmented polymers at 12 months bottle age may give an indication of the colour stability of the wine. Alternately, an increase in anthocyanin extraction in the presence of tannins may involve the preferential binding of tannins with the anthocyanin pigments rather than with pulp polysaccharides (Bindon, 2013a). This would bring about a change in the equilibrium between free and polymerised anthocyanins in the wine solution promoting greater extraction of colour pigments from the skin.

By artificially increasing the sink for tannin adsorption with a double complement of pulp components, a greater amount of tannin was removed. Based on the results of the tannin compositional analysis presented in Table 4.4, it would be expected that the residual tannin in whole-berry ferments was primarily derived from the skin.

The effect of seed components on anthocyanin concentration in the wine was highlighted by the corresponding development of non-bleachable pigment. Wine made with a double complement of either skins or seed showed similar development of non-bleachable pigments to the control wine at bottling. However by 12 months bottle age, wine with a double complement of skins had significantly higher (70%) non-bleachable pigment concentrations than the control wine, while wine with a double complement of seeds showed the opposite result, non-bleachable pigment being 68% lower than in the control wines.

Therefore, in order to prolong colour stability in tannin-poor Pinot Noir wines, it may be important to differentially extract and retain skin tannins early in the wine-making process. However the implications of imposing such a procedure at a commercial level would be considerable and the investigation of a more practical solution is recommended. This study also showed that the interaction between skin and seed components to promote both anthocyanin and tannin extraction was accentuated in the absence of pulp.

4.6 CONCLUSION

Making wine from Pinot Noir grapes has long been a challenge due to the delicate flavour, light colour and sometimes poor ageing potential of the wine. We have demonstrated that the interaction of tannin derived from the skins of Pinot Noir grapes with free anthocyanin to form pigmented tannin may have a significant impact on the long term stability of Pinot

Noir wine colour. The study found that the delay in the formation of pigmented tannin from seed derived tannin coincided with a later release of the seed tannin into the wine matrix, while the presence of either skin or seed tannin in excess, promoted the release of colour pigment from the skin. Pulp components appeared to reduce the concentration of seed tannins, but had far less effect on skin tannins. While maceration techniques that promote greater release of tannin and pigment from the pomace have traditionally been used to make Pinot Noir wines, we have shown that it is important to consider the nature and source of the tannins extracted. Certainly, this study has shown that when seed-derived tannins (or possibly other seed phenolics) are in excess, wine colour development was significantly compromised. The results also show that tannin concentration and composition have roles in the formation of stable, non-bleachable colour in Pinot Noir. The presence of seeds during fermentation was shown to enhance the formation of non-bleachable colour pigments, except when seeds were present in excess. In the Pinot Noir samples studied, seed tannin was more strongly adsorbed by pulp cell wall material than skin tannin, and as such the composition of finished wines more closely reflected that of skin tannin. Consequently, the enhanced development of non-bleachable pigment when seeds were present during fermentation may be due to the interaction of anthocyanin with seed phenolics other than tannin, and further research in this area is warranted. Traditionally, the application of extended maceration or the addition of oenotannins, have been used to make Pinot Noir wines. Techniques such as the addition of exogenous grape skin or seeds were recognised as possible alternatives in the augmentation of wine tannin. While the extra complement of seeds had the most significant effect on the concentration of wine tannin, it had a negative effect on the formation of non-bleachable pigments. The extra complement of skins was a more promising approach, as it contributed additional

non-bleachable colour to the wines, yet the increase in tannin was small relative to seed addition. In conclusion, the identification of techniques which enhance colour and skin tannin extraction and retention in Pinot Noir winemaking is an important focus for future research

4.7 SUBSEQUENT RESEARCH FOCUS

This study demonstrated that seed tannin had a significant role in the formation of stable, non-bleachable colour in Pinot Noir and that it was more susceptible to adsorption by cell wall components of the grape pulp than were skin tannins. However when present in excess seed phenolic compounds were found to compromise colour stability. Augmenting the extraction of both colour pigments and tannin from Pinot Noir grape skins may therefore be beneficial to long term colour stability of the wine. The next chapter investigates the potential for readily available exogenous grape solids to be used as a source of oenotannins and evaluates their role in modifiying the phenolic profile of Pinot Noir wines.

5

THE EFFECT OF SUPPLEMENTARY TANNINS ON THE PHENOLIC COMPOSITION OF PINOT NOIR WINES

Experiments in this chapter were presented as a defended poster at 15th Australian Wine Industry Technical Conference Sydney, Australia, July 2013.

Title: *'Waste not, want not': can winery by-products be recycled to improve wine quality?*

Authors: *Angela M. Sparrow, Robert G Dambergs and Dugald C Close*

5.1 ABSTRACT

Exogenous sources of tannins were added to *Vitis vinifera* cv. Pinot Noir ferments in order to improve the colour attributes of the wine. Pigments and tannins in wines made from traditional Pinot Noir ferments were compared with ferments to which fresh grape pomace of either Pinot Noir, Pinot Gris or Chardonnay grapes were added. Also compared was the addition of either a commercial liquid grape skin tannin (LGST) extract or fermented grape marc. A second experiment compared wines made with the addition of the fresh skins of the same three grape varieties, while a third experiment compared fresh and fermented skins of Pinot Noir as sources of exogenous grape phenolics. When analysed at six months bottle age, Pinot Noir marc was shown to reduce the stable pigment (resistant to sulphur dioxide bleaching) content of wine by 29%, whereas the addition of 20 mL/L LGST increased stable pigment 57%. The addition of either fresh or fermented Pinot Noir grape skins however was found to increase stable pigment by 21%. Neither Chardonnay pomace nor skins had a significant impact on stable pigment. This investigation showed that the addition of exogenous tannins to fresh grape musts may be suitable in some instances but the outcome depends on whether skin or seed tannins are included, as seed derivatives appear to compromise the development of stable pigment.

Skin based tannins were found to be more effective at producing stable colour as well as improving colour density and wine hue.

5.2 INTRODUCTION

Anthocyanin pigments and tannins are major components of red wine and their relative concentrations have been correlated with measures of red wine quality (Cozzolino, et al. 2008, Holt, et al. 2008, Kassara and Kennedy 2011). The effectiveness of their extraction during winemaking depends on their location in the berry and their solubility (Cortell et al., 2005, Adams, 2006). The different properties, localizations and extractability of skin and seed tannins determine the contribution of tannins from each tissue source and have significant effect on wine properties (Vidal et al., 2003a, Koyama et al., 2007a). Consequently extraction of skin and seed tannins in optimal proportions is essential for wine quality (Bautista-Ortin et al., 2007).

All phenolic compounds are unstable and undergo numerous enzymatic and chemical reactions during winemaking and ageing. Appearance and sensory changes during red wine ageing have been ascribed to anthocyanin-tannin reactions (Cheynier et al., 2006). The grape variety determines both the amount and the structure of wine flavanols, (Mattivi et al., 2009) leading to differences in the mean degree of polymerization which in turn is thought to influence astringency and bitterness of red wine (Singleton, 1992, McRae et al., 2013). Skin tannins contain epigallocatechin subunits and generally have a low proportion of epicatechin 3-O-gallate while seed tannins lack epigallocatechin and contain a comparatively high proportion of epicatechin 3-O-gallate (Kennedy et al., 2001).

Pinot Noir grapes tend to have a low concentration of anthocyanin relative to other red wine varieties and these are of a more stable, non-acylated form (Heazlewood, et al. 2006,

Mazza, et al. 1999). In addition, they have an unusual distribution of tannins, with seed tannins being higher in concentration than skin tannins (Mattivi, et al. 2009, Kennedy 2008, Downey, et al. 2003).

To balance these characteristics of Pinot Noir grapes exogenous sources of grape phenolics are sometimes employed to improve the quality and stability of Pinot Noir wines.

However, food processing legislation places constraints on the addition of exogenous compounds to wines and this is supported by the market demand for products containing only locally produced raw materials. Meanwhile, many commercial wineries produce a number of different wine styles made from different grape varieties, and a range of grape solids are produced as by-products. In the case of white or sparkling wines, fresh juice is pressed off the grapes prior to fermentation leaving the fresh grape solids (pomace, composed of skins, pulp and seeds), whereas grape marc (fermented skins and seeds) is a common waste product of red wine production. While these by-products are normally composted, sent off-site for tannin extraction or for use as stock-feed, both are readily accessible sources of grape phenolics (Gibson and Grills, 2007). Apart from the anthocyanins present in red grape skins, the phenolic composition of red and white grape skins does not differ to a great extent (Borbalan et al., 2003) and so in this study the potential of red and white grape pomace, grape skins, a commercial liquid skin tannin extract, grape marc and fermented grape skins were assessed for their suitability to supplement the polyphenolic profile of Pinot Noir wines.

Grape pomace contains skins, seeds and pulp and is relatively easy to prepare, however in order to investigate the relative contribution of seeds and skins to the wine tannin composition, the effect of skins alone was also examined by removing skins from the berries thereby mitigating the effects of grape pulp and seeds, as described in Chapter 4.

Acknowledging that in commercial practice it would be much simpler to separate fermented skins from grape marc, a further experiment compared the use of fermented grape skins with fresh grape skins. Such detailed comparisons as described in this study were made possible using microvinification techniques (Dambergs and Sparrow, 2011, Smart et al., 2012, Sparrow et al., 2013a, Sparrow et al., 2014), which reflect the dynamic changes that take place in the wine matrix during fermentation.

5.3 MATERIALS AND METHODS:

The grape varieties chosen as supplementary sources of phenolic compounds for this study, were those that are readily available in a cool climate region at the same time that Pinot Noir is harvested. Pinot Gris is a genetic mutant of Pinot Noir distinguished by having only one skin cell layer that contains colour pigments rather than the two cell layers found in Pinot Noir (Lecas and Brillouet, 1994). Chardonnay grapes have no red pigment but are a source of both skin and seed tannins (Yilmaz and Toledo, 2004).

5.3.1 Grape sampling and replication

Grapes of *Vitis vinifera* cv. Pinot Noir were used as the base must for each of the three trials described; *Vitis vinifera* cv. Pinot Gris and *Vitis vinifera* cv. Chardonnay grapes were used as additional sources of grape solids. The grapes were harvested from a vineyard in northern Tasmania in April 2012. For each of the trials described, four replicates of each treatment were included. For each replicate, 100 berries from each Pinot Noir clone and cultivar were selected at random to determine berry mass and fruit composition. Grape juice total soluble solids (°Brix) were measured using a hand-held refractometer; the pH of the juice was measured with a Metrohm pH meter/autotitrator and titratable acidity

measured by titration with 0.333 M NaOH to an end point of pH 8.2 (reported as g/L tartaric acid).

5.3.2 Extraction of tannin and colour from grape and marc tissues

Two hundred grams of berries from each clone and variety were frozen at 20 ^{o}C for later analysis of grape colour (Iland et al., 2004) and tannin (Dambergs et al., 2012b). In order to compare the tannin concentration of grape marc with that of fresh grapes, 40 g of Pinot Noir marc was reconstituted in 110 mL model wine solution (saturated solution of potassium hydrogen tartrate in 12% (v/v) ethanol. The reconstituted marc was extracted and analysed for colour and tannin using the same methods as for whole grapes (Iland et al., 2004, Dambergs et al., 2012b).

5.3.3 Treatments for winemaking

Ten kilograms of Pinot Noir grapes (Clone 115) from each of four replicates were divided into six 1 kg batches and four 200 g batches. The 1 kg batches were co-fermented with fresh pomace and the 200 g batches were co-fermented with fresh skins.

Trial A: Co-fermentation with fresh or fermented grape solids or liquid grape skin tannin:

Five kilograms each of Pinot Noir (clone 115), Pinot Gris and Chardonnay grapes were harvested on the same day as 40 kg of Pinot Noir clone 115 used in the base must. Fresh grape pomace was prepared from each grape variety (Pinot Noir, Pinot Gris and Chardonnay) by removing stalks from 1 kg of grapes and hand pressing the berries in a basket press to recover 500 ml of juice. The fresh grape solids were removed from the press and 10% (w/w) used in the co-fermentation trials. The grape pomace additions were equivalent to 200 g of fresh grapes. In addition to the fresh pomace additions, this trial compared the addition of a commercial liquid grape skin tannin extract (LGST) at the

recommended rate (20 mL/L of must) while a sixth treatment used fermented Pinot Noir marc (8% w/w; equivalent to 200 g of fresh grapes) as a source of grape phenolics.

For each replicate of six treatments, Pinot Noir grapes were de-stemmed by hand to yield 1 kg of berries that were hand-crushed then transferred to a 1.5 L Bodum® coffee plunger for fermentation. Treatments for this trial were: (1) Pinot Noir (PN) must with no additions (control); (2) Pinot Noir must with 20% (w/w equivalent) additional Pinot Noir pomace; (3) Pinot Noir must with 20% (w/w equivalent) Pinot Gris (PG) pomace; (4) Pinot Noir must with 20% (w/w equivalent) Chardonnay (CH) pomace; (5) Pinot Noir must with 20 mL/L LGST and (6) Pinot Noir must with 20% (w/w equivalent) Pinot Noir marc.

Trial B: Co-fermentation with fresh grape skins:

To investigate the relative contribution of seeds and skins to the wine tannin composition, the same sources of grapes as described in Trial A (Pinot Noir, Pinot Gris and Chardonnay) were used, however in this experiment only the skins of each cultivar were added to the Pinot Noir base must. Four replicates of each treatment were prepared: skins from 40 g of berries of the selected grape cultivar were removed from the grapes by hand and added to 200 g of Pinot Noir must. Wines made with additional grape skins were compared with wine from a Pinot Noir ferment having no additional skins. Treatments for this second trial were: (1) Pinot Noir must with no additions (control); (2) Pinot Noir must with 20% (w/w equivalent) additional Pinot Noir skins; (3) Pinot Noir must with 20% (w/w equivalent) Pinot Gris skins and (4) Pinot Noir must with 20% (w/w equivalent) Chardonnay skins.

Trial C. Co-fermentation with fresh or fermented Pinot Noir skins:

As the practical implications of removing fresh grape skins on a commercial scale appear quite daunting, a third experiment was designed to compare the properties of wine made with either fresh or fermented grape skins, the latter being more readily separated from

the seeds. Skins were isolated from either fresh Pinot Noir grapes or Pinot Noir grape marc and added to a base Pinot Noir grape must. Six kilograms of grapes of Pinot Noir (Clone G5V15) were harvested and the fruit divided into four replicate samples of 1.5 kg with 200 g sub-samples taken for fruit composition analysis as described above. Fruit from each replicate was randomly divided into four 200 g batches. The fresh skins were removed from a further 40 g of Pinot Noir grapes and added to 200 g of Pinot Noir must; 14 g of fermented skins (equivalent to 40 g of fresh grapes) were separated from grape marc and added to a second 200 g of Pinot Noir must. Treatments for the third trial were: (1) Pinot Noir must with no additions (control); (2) Pinot Noir must with 20% (w/w) additional fresh Pinot Noir skins; (3) Pinot Noir must with 20% (w/w) fermented Pinot Noir skins.

5.3.4 Preparation of Pinot Noir grape marc

To prepare the grape marc and fermented skins used in trials A and C, 10 kg of Pinot Noir grapes Clone D5V12/D4V2 were harvested one week prior to the winemaking trials. Grapes were crushed in a Marchisio Grape Crusher/Destemmer (1000 to 1500 kg/h) and fermented to dryness in a 20 L food grade plastic bucket using submerged cap maceration. The end of fermentation was confirmed at less than 2 g/L of residual sugar using Clinitest™ reagent tablets (Bayer Australia Ltd.). The wine was pressed in a flatbed press at 200 kPa of pressure, and produced 4 kg of grape marc.

5.3.5 Microvinfication protocol

Wine was made using submerged cap micro-fermentation techniques (Dambergs and Sparrow, 2011, Smart et al., 2012). To each fermentation vessel 50 mg/L SO_2 was added in the form of potassium metabisulphite and the 'musts' refrigerated overnight at 4^{o}C. The following day the grape must preparations were allowed to equilibrate at 25^{o}C then

inoculated with 300 mg/L RC212 yeast solution and fermented at 25°C (±1°C). On day 3 of the fermentation, 300 mg/L of diammonium phosphate was added to each fermentation vessel to provide 60 mg/L yeast assimilable nitrogen. Wines were fermented to dryness which was confirmed by testing for residual sugar using Clinitest™ tablets; all wines were found to be dry (< 2 g/L residual sugar). For the 1 kg ferments, conducted in 1.5 L Bodum™ coffee plungers, wine was pressed by depressing the plunger to a specified mark on the vessel and 500 mL of wine recovered. For trials B and C that used 250 mL fermentation vessels, the wine was pressed using a plunger with a mesh sieve (mesh size 1mm x 1 mm) fixed at the base to recover 100 mL of wine. The wines were cold settled at 4°C for two weeks then racked into amber glass bottles. After 4 weeks storage at 12°C the wine was bottled under CO_2 cover. A 10 mL sample of the wine was taken for phenolic analysis at bottling. Following six months storage at 12°C a second sample from each wine was taken for phenolic analysis.

5.3.6 Phenolic analysis by spectroscopy

Wines were allowed to equilibrate at room temperature and clarified by centrifugation at 5,000 rpm for five minutes using an Eppendorf 5417C centrifuge with an Eppendorf F45-30-11 rotor (Crown Scientific, Australia). UV-Visible spectrophotometric analysis was conducted using a Genesys™ 10S UV-Vis Spectrophotometer calibrated for rapid measurement of methyl cellulose precipitable tannins (Dambergs et al., 2012b), in conjunction with the Modified Somers Assay described by Mercurio et al. (2007). Total phenolics, total pigment (free and bound pigments), free anthocyanin, pigment resistant to sulphite bleaching (non-bleachable pigment), percentage non-bleachable pigment, colour density, hue and sulphite resistant hue (hue S02) of the wines at pH 3.4 and total tannins at pH 0.0, were calculated at bottling and again at six months bottle age. Wine samples

were diluted in each of three solutions (Assay A: 0.1% (v/v) acetaldehyde in model wine buffer; Assay B: 0.375% sodium metabisulfite in model wine buffer and Assay C: 1M hydrochloric acid). For all assays, the samples were mixed by inverting screw capped tubes three times and incubated in the dark at room temperature. Wine samples analysed using Assays A and B were incubated for 1 h at room temperature. Assay C samples were incubated at room temperature in the dark for 3 h. Absorbances of all samples were read at 2 nm intervals from 200 to 600 nm using an Ultraviolet-Visible (UV-Vis) Spectrophotometer (Model Genesys™ 10S Thermo Fisher Scientific Inc., Madison, WI, USA) that had been calibrated for rapid measurement of methyl cellulose precipitable tannins by UV-VIS spectrophotometry and chemometrics (Dambergs et al., 2012b). Wine phenolic parameters calculated from these assays were free anthocyanin, total tannin, non-bleachable pigment, colour density, hue and hue SO2. Free anthocyanin refers to anthocyanins in solution that have not become chemically bound to other molecules and appear red at low pH. Non-bleachable pigments are those pigments that are resistant to bleaching in the presence of sulphur dioxide and consist of either polymerised anthocyanin molecules (pyranoanthocyanins) (Boulton, 2001, Cheynier et al., 2006) or molecules in which one or more anthocyanin molecules have become bound to proanthocyanidins (condensed tannins) to form 'pigmented tannins' (Harbertson et al., 2003). Wine hue is calculated from the ratio of absorbance of the sample at 420 nm to its absorbance at 520 nm in model wine solution containing 0.1% (v/v) acetaldehyde and generally increases with age as free anthocyanins become bound within pigmented tannins, to form red-brown pigments (Somers, 1971). Hue SO2 is calculated from the ratio of the absorbance of the sample at 420 nm to its absorbance at 520 nm in model wine solution containing 0.375% sodium metabisulphite and is a measure of the hue of the wine pigment resistant to

sulphur dioxide bleaching; a low hue SO_2 is indicative of stable colour shifted towards the blue-purple spectrum, as opposed to red-orange, and may be indicative of a larger proportion of flavanyl-vinyl-pyranoanthocyanins (portisins) which are blue-purple in colour (Alcalde-Eon et al., 2006, Cheynier et al., 2006).

5.3.7 Proanthocyanidin (PA) composition of wines

The proanthocyanidin (PA) composition of wines from Trial A was determined at six months bottle age. Tannin concentration was determined using the methyl cellulose precipitable tannin assay (MCPT) (Sarneckis et al., 2006). A 4 mL sample of wine was then loaded onto a solid phase extraction (SPE) cartridge, and total tannins were isolated. The isolated tannins were then subjected to acid-catalyzed depolymerization in the presence of excess phloroglucinol (phloroglucinolysis) (Kassara and Kennedy, 2011). Seven tannin composition measures were calculated from the tannins isolated from wine samples: mean degree of polymerisation (mDP) a measure of the mean number of proanthocyanidin subunits in wine tannin polymers; total proanthocyanidin concentration (total PA), following depolymerisation; percent mass conversion (% MC), representing the proportion of the tannin that can be depolymerised by the assay; percentage of tri-hydroxylated subunits (% tri-OH), an indicator of the proportion of skin tannin in the wine, as these subunits are not present in seeds (Souquet et al., 1996); percentage of galloylated subunits (% gall), an indicator of the proportion of seed to skin tannin in wine (as epicatechin-gallate also occurs in skin); ratio of tri-hydroxylated to galloylated PA subunits (tri-OH/gall), an indicator of the proportion of skin to seed tannins in the wine; molecular size at 50% elution by gel permeation chromatography (50% GPC) (Kennedy and Taylor, 2003b) an indicator of the median size of tannin polymers in wine.

The measures mDP, percent trihydroxylation, and percent galloylation can only be applied to proanthocyanidin subunits that have been recovered by phloroglucinolysis and are therefore interpreted relative to mass conversion percentage. The ratio anthocyanins*skin tannins/seed tannins has been proposed as an indicator of wine flavonoid composition, wine colour and wine quality (Ristic et al., 2010). Using the values for subunit composition determined by phloroglucinolysis and the anthocyanin concentration determined by spectroscopy and chemometrics, this wine quality indicator was also determined.

5.3.8 Statistical analysis

Mean and standard deviation for phenolic characters in each trial were calculated using analysis of variance (GenStat Release 13.1 Copyright 2010, VSN International Ltd). For wine assessed at one time point, the phenolic composition of each treatment was analysed using 1-way ANOVA, wines assessed at two-time points used Repeated Measures ANOVA with treatment as the main plot and time as the sub-plot. In each case ANOVA was followed by post-hoc analysis using Fisher's Protected Least Significant Difference (LSD) test.

5.4 RESULTS

5.4.1 Grape composition

The composition of each grape variety used in Trials A and B was determined prior to fermentation (Table 5.1). The berry weight, sugar content and pH were similar for all three varieties. The total phenolic concentration of Pinot Noir for both the pomace addition and base must was significantly higher than for Pinot Gris (PG) and Chardonnay (CH).

Table 5.1 Composition of fruit and exogenous phenolic sources (mean and standard deviation, n=4)

Berry composition	Pinot Noir base must Clone 115	Pinot Noir pomace Clone 115	Pinot Noir Clone D5V12/D4V2	Pinot Noir Clone G5V15	Pinot Gris pomace	Chardonnay pomace	Grape skin tannin extract	Pinot Noir marc Clone D5V12/D4V2 (reconstituted)
Berry Wt (g)	1.53 ± 0.1 a	1.33 ± 0.1 b	†1.29	1.27 ± 0.0 b	1.38 ± 0.1 b	1.43 ± 0.1 b	NA	NA
Sugar (°Brix)	22.2 ± 0.4 b	21.5 ± 0.4 b	†22.4	21.7± 0.3 b	23.0 ± 0.3 a	20.9 ± 0.1 b	¥ <2.5	22.4
pH	3.37 ± 0.1 a	3.30 ± 0.1 a	†3.15	3.33 ± 0.1 a	3.25 ± 0.0 a	3.19 ± 0.0 b	¥ <3.3 a	3.15
Titratable acidity (g/L)	11.4 ± 0.2 a	9.13 ± 0.1 b	†11.6	10.2 ± 0.1	8.39 ± 0.3 c	8.65 ± 0.3 bc	¥ <5.0	11.6
Anthocyanin (mg/g)	0.63 ± 0.1 b	0.69 ± 0.1 b	0.61 ± 0.2 b	0.68 ± 0.01 b	0.12 ± 0.0 c	‡0.06 ± 0.01 d	4.96 ± 1.2 a	0.14 ± 0.0 c
Total tannin (mg/g)	7.03 ± 0.4 b	8.68 ± 1.2 a	7.82 ± 1.9 a	6.84 ± 0.30 b	6.87 ± 0.5 b	5.16 ± 0.6 c	49.4 ± 13.0 d	5.21 ± 1.2 c
Total phenolics (AU/g)	1.23 ± 0.1 b	1.42 ± 0.2 b	1.29 ± 0.3 b	1.23 ± 0.05 b	1.06 ± 0.1 c	0.83 ± 0.1 d	1129 ± 0.49 a	0.83 ± 0.2 d

†Unreplicated sample; ‡Background absorbance at 520 nm; ¥Manufacturer's specifications; NA = not applicable; numbers in each row having the same letter are not significantly different.

This was not simply a reflection of the anthocyanin content of Pinot Noir relative to the other varieties, as there was also a large difference in the total tannin content of the grapes. Pinot Noir grapes used for the pomace addition were 14% higher in tannin than Pinot Gris and 48% higher in tannin than Chardonnay grapes. The fresh Pinot Noir grapes used to prepare grape marc contained 7.8 mg/g of tannin and when reconstituted in model wine solution with the equivalent volume of liquid to that of fresh grapes, the grape marc contained 5.2 mg/g of tannin indicating that approximately 66% of the grape tannin remained in the grape marc after the initial fermentation. Similarly, approximately 23% of the fresh grape anthocyanin remained in the grape marc.

5.4.2 Changes in phenolic composition from bottling to six months bottle age

Trial A: Co-fermentation with grape pomace, LGST or PN grape marc:

Interactions were observed between exogenous phenolic sources and bottle age for all but one of the phenolic parameters examined in this study; only wine hue showed no significant interaction (Table 5.2). For each of the fresh pomace varieties added to the must, there was an average increase in total phenolics of 11% at bottling, with LGST and Pinot Noir marc additions resulting in a 19% increase. However at six months bottle age the total phenolic concentration of wines with added PN pomace or CH pomace were not significantly different from the control wines, suggesting some loss of phenolics from the wine during the first six months in the bottle. When considering individual phenolic parameters, as might be expected, significant increases in total pigment were observed, except for those treatments with little or no additional pigment source (control, PG pomace and CH pomace). However at six months bottle age the total pigment concentration of wine with added Pinot Noir pomace was no longer significantly different from the control wines.

Table 5.2 Wine phenolics for six pomace treatments at 50 and 230 days post-inoculation

Phenolic character	Must addition						
	Nil (control)	PN pomace	PG pomace	CH pomace	LGST	PN marc	*P-value*
	Wine at 50 days post-inoculation (bottling)						
Total phenolics (AU)	37.8 c	44.0 ab	42.2 b	43.2 b	46.3 a	44.3 a	*<0.001*
Total pigment (AU)	16.1 c	19.3 b	17.5 bc	16.9 bc	23.0 a	18.8 b	*<0.001*
Anthocyanin (mg/L)	310 c	372 b	337 bc	325 bc	441 a	362 b	*<0.001*
NBP [†] (AU)	0.36 b	0.40 b	0.38 b	0.38 b	0.60 a	0.40 b	*<0.001*
% NBP[†]	2.25 b	2.09 b	2.12 b	2.27 b	2.60 a	2.14 b	*0.001*
Tannin (g/L)	0.71 b	0.91 a	0.85 a	0.92 a	0.93 a	0.92 a	*0.031*
Colour density (AU)	3.73 c	4.27 b	3.98 bc	3.93 bc	5.57 a	4.35 b	*<0.001*
Hue (AU)	0.63 a	0.62 ab	0.62 ab	0.63 a	0.59 b	0.60 ab	*0.019*
Hue SO_2	1.81 a	1.85 a	1.84 a	1.86 a	1.52 c	1.76 b	*<0.001*
	Wine at 230 days post-inoculation (6 months bottle age)						
Total phenolics (AU)	36.0 c	36.2 c	39.5 b	37.9 bc	44.9 a	43.7 a	*0.004*
Total pigment (AU)	12.6 c	13.2 c	13.6 c	12.4 c	18.9 a	15.7 b	*0.018*
Anthocyanin (mg/L)	234 c	242 c	253 c	229 c	347 a	295 b	*0.017*
NBP[†] (AU)	0.58 b	0.63 b	0.58 b	0.57 b	0.91 a	0.56 b	*0.01*
% NBP [‡]	4.60 a	4.81 a	4.27 ab	4.64 a	4.87 a	3.57 b	*0.008*
Tannin (g/L)	0.69 a	0.67 a	0.79 ab	0.77 ab	0.92 b	0.92 b	*0.008*
Colour density (AU)	4.10 b	4.65 b	4.30 b	4.26 b	6.03 a	4.41 b	*0.039*
Hue (AU)	0.73 a	0.72 a	0.71 a	0.73 a	0.66 a	0.68 a	*0.368*
Hue SO_2[§]	1.52 a	1.56 a	1.53 a	1.62 a	1.33 b	1.59 a	*0.026*

Mean (n=4); [†]NBP, non-bleachable pigment; [‡]% NBP (NBP **/** total pigment)*100; [§]Hue SO_2, ratio of absorbance at 520nm/420nm in model wine plus 0.375% sodium metabisulphite; PN, Pinot Noir; PG, Pinot Gris; CH, Chardonnay; for each time period, numbers within a row with different letters are significantly different at $P \leq 0.05$.

For each treatment there was an average increase of 28% in wine tannin at bottling relative to the control treatment, whereas at six months bottle age, there was no significant difference in tannin concentration between control wines and those made with additional fresh pomace (Table 5.2). The anthocyanin content of wine made with additional PN pomace was 20% higher than the control at bottling and wines with added PN marc were 17% higher in anthocyanin concentration, while the increase observed with the addition of LGST was 42%. After six months in the bottle, the anthocyanin content of the control wines and those with added fresh pomace had declined by an average of 40% regardless of the source of grape variety from which the pomace was derived. By contrast the anthocyanin content of wine with added PN marc had declined by 23% while those with added LGST had declined by 27%. As noted below the reduction in anthocyanin is most likely to be a consequence of the formation of polymeric pigments or anthocyanin-tannin adducts as the wine matured.

At bottling the non-bleachable pigment (NBP) content of treated wines was not significantly different from the control with the exception of those with added LGST which were 67% higher in NBP than the control wines. While the average increase in NBP for the control and all of the treatment wines was 52% from bottling to six months bottle age, the only wine that remained significantly higher (57%) than the control wines was the LGST treated wine (Table 5.2). Calculation of the percentage of total pigment that was resistant to sulphite bleaching (% NBP) again showed that only the wine with added LGST was significantly higher than the control wine at bottling, which was attributed to the stable colour pigments (5%) originally contained in the liquid grape skin tannin extract (. At six months bottle age there was a two-fold average increase in % NBP for all of the wines, with

the exception of PN marc treated wines which increased 1.7-fold, suggesting that it too had conferred stable pigments on the must by the time the wine was bottled (Table 5.2).

At bottling, the colour density of wines treated with Pinot Noir pomace, PN marc and LGST were significantly higher than the control wines being 15%, 17% and 49% respectively, a feature apparently associated with the additional pigment from these exogenous sources of grape phenolics. The colour density of each wine increased by an average of 8.6% from bottling to six months bottle age with the exception of the treatment to which Pinot Noir marc had been added in which the colour density of the wine remained stable; wine hue increased by an average of 14% across all treatments (Table 5.2).

These results indicate that the tannin contributed by the fresh pomace addition (28%) was lost from the wine matrix in the first six months after bottling and that during this time only the addition of LGST to the base Pinot Noir musts appeared to contribute to the stable pigments.

Examination of the tannin composition of the wines aged for six months in the bottle showed that there was no significant difference in mDP between any of the wines, whereas the percentage of tri-hydroxylated PAs decreased significantly regardless of the source of exogenous phenolics, with PN marc and CH pomace having the greatest impact (Table 5.3). For each treatment wine the percentage of galloylated PAs was significantly higher than the control wines, the highest values being associated with PN pomace, CH pomace and PN marc. The ratio of tri-hydroxylated to galloylated PAs was highest for the control and LGST treatments. Taken together these results indicate that for the treatments with added pomace or marc, the tannins that could be depolymerised using phloroglucinolysis were primarily from the seeds.

The ratio of anthocyanins to skin tannins has been reported to indicate a greater availability of skin tannins per unit of anthocyanins potentially resulting in a higher incorporation of anthocyanins into pigmented polymers (Ristic et al., 2010). As a consequence these researchers proposed that higher concentrations of anthocyanins and skin tannins in berries, coupled with a lower concentration of seed tannins were associated with higher wine quality. The anthocyanin content determined by spectroscopy (Table 5.2), together with the concentration of trihydroxylated and galloylated PA subunits (Table 5.3), were used to calculate a wine quality indicator as proposed by Ristic et al. (2010) using the ratio:

WQI= anthocyanins *skin tannins /seed tannins

The wine quality indicator (WQI) assumes that the tannins which are depolymerisable by phloroglucinolysis assay represent the tannins that have not become bound into the structure of condensed tannins. However a high mass conversion percentage (%MC) indicates a large proportion of tannins had become condensed and cannot be depolymerised by the assay. Only the wines with added LGST had a low %MC and WQI that was significantly higher than the controlTreatments with added pomace or marc had significantly lower WQI than both LGST wines and the control, indicating that the latter exogenous tannin sources had a higher proportion of unstable depolymerisable tannins sourced from the seed (Table 5.3). The PAs with greatest molecular mass were those from the PG and CH pomace treatments, the remainder not being significantly different from the control wines (Table 5.3). The assumption, based on the WQI, that the addition of fresh pomace to Pinot Noir musts conferred a higher proportion of seed tannin than skin tannin to the wine was tested in Trial B where fresh grape skins only (no pulp or seeds) of the same grape varieties were added to Pinot Noir musts.

Table 5.3 Proanthocyanidin composition of wine (at six months) made from musts with added grape pomace, grape skin tannin extract or grape marc.

Must addition	mDP[‡]	Total PA g/L[ϰ]	%MC[§]	%tri-OH[φ] PA	%gall[Ђ] PA	tri-OH/ gall[#]	50% GPC Tannin[ж]	WQI[¥]
Nil (control)	7.70	5.81 a	60. 6 e	24.9 a	2.53 e	9.84 a	1731 b	2.32 b
20% PN pomace	7.42	7.63 a	67.2 c	23.4 b	2.88 b	8.13 b	1671 b	1.97 b
20% PG pomace	7.47	7.25 a	64.1 d	22.5 d	2.70 d	8.33 b	1951 a	2.16 b
20% CH pomace	6.88	7.68 a	72.0 b	21.9 e	2.82 c	7.77 b	1765 a	1.78 b
20ml/L LGST	6.97	4.68 b	49.1 f	23.2 c	2.22 f	10.4 a	1561 b	3.67 a
20% PN marc	6.23	7.22 a	72.9 a	19.5 f	3.04 a	6.44 b	1563 b	1.90 b
P-value	*0.07*	*0.02*	*0.04*	*0.03*	*0.002*	*0.01*	*0.01*	*<0.001*
LSD (Trt)	NS	1.91	0.15	0.03	0.003	2.07	215	0.76

[‡]mDP, Mean degree of polymerisation; [ϰ]Total Proanthcyanidin Subunits after depolymerisation; [§]MC, Mass conversion = % recovery of tannin subunits by phloroglucinolysis based on the MCPT tannin concentration (in g/L); [φ]% Trihydroxylated subunits; [Ђ] % galloylated subunits; [#]ratio of trihydroxylated to galloylated subunits; [g]molecular size at 50% GPC (g/mol); [¥]WQI, Wine Quality Indicator: Anthocyanin concentration*%trihydroxylated PA/%galloylated PA. PN, Pinot Noir; PG, Pinot Gris; CH, Chardonnay; Numbers within a column with different letters are significantly different at P ≤ 0.05. NS, Not significant at P ≤ 0.05.

Trial B: Co-fermentation with grape skins:

While the must size for Trial B was smaller than for Trial A, the parameters of phenolic composition of the control wines from each ferment size were not significantly different at P≤0.05 (Tables 5.2 and 5.4 and Chapter 2). When 20% (w/w) of isolated skins from either Pinot Noir, Pinot Gris or Chardonnay grapes were added to Pinot Noir base must, the only

parameter that showed a significant interaction between grape skin addition of any variety and bottle age, was non-bleachable pigment (P=0.003) (Table 5.4), demonstrating that the development of non-bleachable pigment during bottle ageing was dependent on the grape variety from which the added skins were sourced. There were however significant effectson other phenolic attributes of the wine determined by the varietal source of the grape skins. At bottling, significant differences between treatments were observed for total phenolics, total pigment, tannin and colour density. Six months after bottling, significant differences between treatments were observed for total phenolics, non-bleachable pigment, tannin, and hue SO_2 (Table 5.4).

The addition of grape skins to Pinot Noir must resulted in an average increase in total phenolics of 7% and an increase in tannin concentration of 26% in the wine at bottling and was independent of the grape variety. The addition of PN skins significantly increased the colour density (7.5%) at bottling but had no effect on the other pigment related parameters. At six months bottle age, wines prepared with either added PN or PG skins maintained an average phenolic concentration 8% higher than the control and a tannin concentration 27% higher than the control, while the wines with added Chardonnay skins were not significantly different to the control wines for these parameters.

Table 5.4 Mean (n=4) Pinot Noir wine phenolics at 50 and 230 days post-inoculation for Pinot Noir musts with added grape skins.

	Must addition				
	Nil (control)	PN skins	PG skins	CH skins	*P-value*
Phenolic character	*Wine at 50 days post-inoculation (bottling)*				
Total phenolics (AU)	33.8 b	36.8 a	36.0 a	35.1 a	*0.046*
Total pigment (AU)	15.7 a	16.8 a	15.4 b	14.9 b	*0.048*
Anthocyanin (mg/L)	300 a	321 a	294 b	284 b	*0.055*
NBP[†] (AU)	0.41	0.44	0.44	0.4	*0.227*
% NBP[†]	2.66	2.63	2.81	2.71	*0.692*
Tannin (g/L)	0.48 b	0.63 a	0.61 a	0.58 a	*0.015*
Colour density (AU)	3.85 b	4.14 a	3.84 b	3.68 b	*0.025*
Hue (AU)	0.65	0.66	0.66	0.67	*0.253*
Hue SO_2	1.7	1.72	1.69	1.76	*0.327*
	Wine at 230 days post-inoculation (6 months bottle age)				
Total phenolics (AU)	35.3 b	39.4 a	36.7 a	35.6 b	*0.048*
Total pigment (AU)	12.9 a	15.0 a	11.7 b	12.5 a	*0.126*
Anthocyanin (mg/L)	224	250	195	222	*0.315*
NBP (AU)	1.03 b	1.46 a	1.20 b	0.84 b	*0.01*
% NBP [†]	8.11 a	9.94 a	10.2 a	6.90 b	*0.139*
Tannin (g/L)	0.62 b	0.83 a	0.75 a	0.68 b	*0.004*
Colour density (AU)	5.85 a	6.15 a	6.06 a	4.85 b	*0.151*
Hue (AU)	0.73	0.73	0.74	0.75	*0.412*
Hue SO_2	1.18 b	1.12 b	1.11 b	1.30 a	*0.044*

NBP, non-bleachable pigment, PN Pinot Noir; PG Pinot Gris; CH, Chardonnay; [†]NBP, non-bleachable pigment; for each time period, numbers within a column with different letters are significantly different at $P \leq 0.05$.

The non-bleachable pigment content of wines with added PN skins was 42% higher than control wines, whereas neither PG nor CH skins had a significant impact on non-bleachable pigment content: a testament to the reduced pigment status of these varieties.

The results of this trial indicated that fresh PN and PG skins had a greater impact on tannin composition of wines co-fermented with Pinot Noir than did CH skins.

Trial C: Comparison of fresh and fermented Pinot Noir grape skins:

Interactions between exogenous Pinot Noir skin additions and bottle age were observed for: non-bleachable pigment (P=0.0375), % non-bleachable pigment (P=0.035) and hue (P=0.005) . In addition, significant effects of each skin type were observed at each time period (Table 5.5). At bottling significant differences between treatments were observed for all phenolic parameters with the exception of hue SO_2. The most notable difference between treatments was a 50% increase in tannin when either fresh or fermented skins were added to the base Pinot Noir must. Observed increases included total pigment: 21% with fresh skin addition, 11 % with fermented skin addition, which were in parallel with increases in anthocyanin concentration. The treatment to which fermented skins were added appeared not to confer a higher concentration of non-bleachable pigment directly to the wine assessed at bottling (Table 5.5).

Table 5.5. Mean (n=4) Pinot Noir wine phenolics at 50 and 230 days post-inoculation for Pinot Noir musts with added fresh or fermented PN grape skins.

	Must Addition			
	Nil (control)	Fresh PN skins	Fermented PN skins	*P-value*
Phenolic character	*Wine at 50 days post-inoculation (bottling)*			
Total phenolics (AU)	30.8 b	35.8 a	35.1 a	*0.010*
Total pigment (AU)	9.41 c	11.4 a	10.4 b	*0.004*
Anthocyanin (mg/L)	178 c	216 a	197 b	*0.004*
NBP (AU)	0.30 b	0.35 a	0.31 b	*0.006*
% NBP	3.16	3.07	3.01	*0.090*
Tannin (g/L)	0.48 b	0.72 a	0.73 a	*0.009*
Colour density (AU)	2.65 b	3.12 a	2.84 b	*0.008*
Hue (AU)	0.76 a	0.74 ab	0.71 b	*0.012*
Hue SO_2	1.94	1.90	1.87	*0.153*
	Wine at 230 days post-inoculation (6 months bottle age)			
Total phenolics (AU)	31.9 b	36.7 a	35.8 a	*0.002*
Total pigment (AU)	8.3 b	10.1 a	9.0 b	*0.005*
Anthocyanin (mg/L)	149 b	181 a	161 b	*0.006*
NBP (AU)	0.48 b	0.60 a	0.56 a	*0.013*
% NBP	5.75	5.93	6.25	*0.190*
Tannin (g/L)	0.60 b	0.83 a	0.83 a	*0.003*
Colour density (AU)	2.99 b	3.62 a	3.37 a	*0.005*
Hue (AU)	0.85 a	0.80 b	0.81 b	*0.025*
Hue SO_2	1.59	1.47	1.43	*0.15*

NBP, non-bleachable pigment, PN, Pinot Noir; PG, Pinot Gris; CH, Chardonnay; for each time period, numbers within a column with different letters are significantly different at $P \leq 0.05$.

At six months bottle age significant differences between treatments were observed for all of the parameters listed above with the exception of percentage non-bleachable pigment and the inclusion of hue SO_2. At this time period, the wines with additional skins, either fresh or fermented, were 38% higher in tannin than the control wines but the increase in tannin concentration from bottling to six months bottle age was less (15% increase) with added grapes skins than for the control wines (50% increase) which may be a consequence of differences in polymerisation reactions in the wine matrix. The colour components of wines with added grape skins were all higher than the control wines, notably, wines to which either fresh or fermented skins had been added had 17% higher colour density and 21% higher non-bleachable pigment concentration than the control wines. In particular, values for hue and hue SO_2 were significantly lower in wines with the addition of either fresh or fermented grape skins suggesting that the more purple hue detected in these wines was likely to remain stable. Blue-purple tints in red wine, that exhibit resistance to sulphite bleaching may indicate the development of a class of pyranoanthocyanins known as 'portisins' (Marquez et al., 2013).

5.5 DISCUSSION

5.5.1 Grape tannin

This study explored options for the addition of easily accessible exogenous sources of grape tannins with the aim of improving the phenolic profile of Pinot Noir wine particular in relation to colour stability. The study highlighted the varietal distinction of Pinot cultivars (Pinot Noir and Pinot Gris) which were shown to be significantly higher in tannin than were Chardonnay grapes.

5.5.2 Co-fermentation with grape pomace, skin tannin extract or grape marc

When Pinot Noir must was co-fermented with 20% fresh pomace, at bottling the wines with added Pinot Noir pomace or Chardonnay pomace had phenolic concentrations that were significantly higher than the control wines, but at 6 months bottle age this difference was no longer apparent, suggesting some loss of wine phenolics from these wines during the first six months in the bottle. From the spectral analysis conducted, the observed change was apparently due to loss of tannin from the wine matrix which may have been caused by the fining of wine tannins by colloidal material or dissolved polysaccharides (Bindon and Smith, 2013b). The loss of wine tannins during bottle ageing where additional grape pulp had been co-fermented with Pinot Noir must was also noted in Chapter 4. The phloroglucinolysis assay demonstrated that the wines made with either fresh Pinot Noir or Chardonnay pomace had a higher percentage of galloylated subunits relative to tri-hydroxylated subunits and that the mass conversion percentage for these wines was also significantly higher than for the remaining treatments signifying that these exogenous sources of phenolic compounds had a higher proportion of depolymerised tannins. As observed in Chapter 4, these findings are consistent with a larger proportion of seed derived tannins in the wine. When the ratio used to estimate wine quality (WQI) as described by (Ristic et al., 2010)) was applied in the current study, only the wines to which LGST had been added had a higher (58%) wine quality indicator than the control wines, whilst the addition of grape pomace of any variety apparently reduced the wine quality indicator by an average of 19%.

Considering that Chardonnay grapes started out with a lower tannin concentration relative to the other grape varieties (Table 5.1), wines with added Chardonnay pomace showed a

relatively high ratio of seed (% galloylated subunits) to skin tannins (trihydroxylated subunits) relative to wines with added Pinot Noir pomace (Yilmaz and Toledo, 2004).

The trial with fresh pomace additions showed that there was also a reduction in the total pigment during the first six months of bottle ageing, which is likely to be associated with ionic and structural changes to anthocyanin pigments with wine age (Boulton, 2001, Cheynier et al., 2006). The observation that co-fermentation with Pinot Noir marc contributed an additional 26% to the concentration of anthocyanin in the wine at six months bottle age demonstrated that a considerable amount of extractable anthocyanins remained in the grape marc after the initial alcoholic fermentation (Somers, 1971, Pinelo et al., 2006) and was extracted in the second alcoholic fermentation. However as the non-bleachable pigment concentration of wines with added Pinot Noir marc was not significantly higher than the control wines, it may be that the higher proportion of seed tannin associated with the grape marc resulted in the formation of NBP that was unstable, as noted in Chapter 4. The effect of seed tannin additions on anthocyanin behaviour in wine has been reported elsewhere (Kovac et al., 1995, Neves et al., 2010) and is investigated in more detail in Chapter 6.

5.5.3 Co-fermentation with grape skins

By comparison with the 28% increase in tannin concentration observed when grape pomace was added to Pinot Noir ferments, the addition of skins alone resulted in a 26% increase in tannin concentration that was independent of the grape variety. At six months bottle age, wines with added PN or PG skins maintained an average tannin concentration 27% higher than the control, which had increased by 50% during six months of bottle ageing. Consequently in wines with added grape skins, the increase in tannin concentration was considerably less (15% increase) suggesting that more tannin

monomers had become polymerised in the presence of additional skins whereas when the extent of polymerisation was less wines (such as in the control wines) loosely associated tannin monomers, dimers and trimers may have become dissociated apparently increasing the tannin concentration. Of particular note is the observation that the wines with added Chardonnay skins were not significantly different to the control wines (Table 5.4) which may be a reflection of the lack of additional pigment associated with the Chardonnay skins which prevented tannin being incorporated in the wine matrix by polymerisation with anthocyanins. Pinot Gris skins did contribute anthocyanins to the grape must, and produced wines which, at six months bottle age, had tannin concentrations that fell between the control wines and those with added Chardonnay skins. This suggests that it was the amount of anthocyanin in the grape must that limited the formation of non-bleachable pigments rather than the amount of skin tannins (Boulton, 2001).

5.5.4 Co-fermentation with fresh or fermented grape skins

In the majority of red wine varieties, most of the phenolic compounds retained in grape marc are from the skins (Pinelo et al., 2006). These phenols are putative antioxidants justifying the use of grape skin as a good source for phenol recovery (Kammerer et al., 2005). The addition of fresh skins to the Pinot Noir (G5 V15) base must resulted in a greater increase in tannin concentration of the wine at bottling than for wine made with PN clone 115 (Trials A and B) and was similar to that observed with the addition of fermented skins for that clone. While some tannin was lost from the wine matrix between bottling and six months bottle age, the tissue source of the tannin was not determined. However, the blue-purple tints in red wine, together with the enhanced colour density and the resistance to sulphite bleaching that developed with bottle age may be attributed to the formation of the class of pyranoanthocyanins known as ‘portisins’ recently described

by Marquez et al. (2013). Portisins or vinylpyranoanthocyanins (pyranoanthocyanin with a vinyl linkage to a flavanol unit) remain blue under acidic conditions. It is possible that additional Pinot Noir skins, either fresh or fermented and LGST, contributed a greater proportion of portisins as suggested by the low hue value of the non-bleachable pigments. Conversely, co-pigmented anthocyanins consist of hydrophobic linkages between anthocyanin moieties and tend to be unstable in the long term, dissociating as the wine ages (Boulton, 2001). Significantly lower values of hue SO_2 suggest that a large proportion of anthocyanins were involved in the formation of stable pigments such as vinyl-flavanyl-pyranoanthocyanins (portisins). However, further experiments to ascertain whether these specific colour compounds were formed in the wines described in this study were beyond the scope of this investigation.

5.6 CONCLUSION

The results indicate that the composition of seed and skin tannins, and the influence of grape pulp as supplements, had a significant impact on the phenolic parameters of the resultant wines. The study emphasised the value of early assessment of the grape phenolic composition and the importance of choosing a maceration technique that maximises the extraction of preferred tannins. The investigation demonstrated that significant benefits to both wine hue and colour stability were derived from the addition of a commercial Liquid Grape Skin Tannin extract and from both fresh and fermented Pinot Noir skins. Whilst grape pomace may be more readily accessible than grape skins, the additional seed tannin within the pomace was found to be detrimental to wine colour stability. Repeated testing of the wines during bottle ageing and analysis of the flavour

and aroma composition of the wines would be advantageous in evaluating wine quality improvements.

5.7 SUBSEQUENT RESEARCH FOCUS

The considerable influence of seed tannins on the phenolic quality of Pinot Noir wines that was demonstrated in Chapter 4 was confirmed in Chapter 5. As seed derived oenotannins are sometimes added to wines with poor phenolic profiles, the effect of seed tannin addition *per se* in Pinot Noir winemaking invited further examination. To complement that examination, access to a naturally occurring seedless clone of Pinot Noir enabling more detailed scrutiny of how Pinot Noir wine colour might develop in the absence of seed derivatives, would be beneficial.

6

6A THE IMPACT OF SEED TANNIN ON COLOUR STABILITY IN PINOT NOIR WINE

Experiment is this chapter were presented as a defended poster at XXVIIth International Conference on Polyphenols, held in conjunction with 8th Tannin Conference, Nagoya, Japan, September 2014.

Title: 'The impact of seed tannins on the phenolic composition of Pinot Noir wine'

Authors: Angela M. Sparrow, Robert G Dambergs and Dugald C Close

6a.1 ABSTRACT

Maceration techniques which promote extraction of colour and tannin are often employed during Pinot Noir winemaking; alternatively, exogenous grape tannins are sometimes added. This study investigated the role of seed tannins in Pinot Noir wine by comparing: (1) seeded and seedless clones of Pinot Noir and; (2) the addition of either fermented seeds or commercial seed tannin product to the must of a seeded Pinot Noir clone. Wine phenolic characters assessed included: anthocyanin, total tannin, non-bleachable pigment, colour density and hue which were estimated during fermentation and at 6 months and 12 months bottle age. At 12 months bottle age, the percentage of pigmented tannin (non-bleachable pigment relative to total pigment) was 2.4-fold higher in the seedless clone than in the seeded clone, consistent with the proposal that a greater proportion of stable pigmented tannins are formed from skin-derived tannins than from seed-derived tannins. The addition of fermented seed or commercial seed tannin preparations resulted in wines that at 12 months bottle age had 43% higher concentration of free anthocyanin and a 35% reduction in pigmented tannin content of the wine relative to control wines. Treated wines also had redder wine hue. These observations were attributed to the competition between seed and skin tannins for binding sites with anthocyanins, the structural arrangement of seed tannin proanthocyanidin polymers, and the stability of the tannin-

anthocyanin complexes. We conclude that winemaking practices that promote seed tannin extraction from Pinot Noir grapes may compromise the quality and ageing potential of the wine.

6a.2 INTRODUCTION

A diverse range of maceration techniques are used in Pinot Noir winemaking to achieve the desired combination of sensory attributes. Wine colour is determined by the amount of anthocyanin extracted from the grape skins during fermentation, the co-pigmentation of these anthocyanins with colourless molecules during winemaking and polymerisation reactions with proanthocyanidins (condensed tannins) to form 'pigmented tannins' (Somers and Evans, 1977, Harbertson et al., 2003, Marquez et al., 2013, Boulton, 2001, Cheynier et al., 2006). In their more oxidised state the flavylium cations of anthocyanins are red in colour whereas the less oxidised quinoidal base can be red or blue-purple, with further reduction causing anthocyanins to become colourless (Ribereau-Gayon et al., 2006, Cheynier et al., 2006, Fulcrand et al., 2006, Scollary, 2010). The Anthocyanin equilibria in red wine is not only a function of pH, alcohol percentage and both the concentration and structure of the co-pigments also have a significant role (Delsart et al., 2012). When present in high concentrations, anthocyanin molecules become associated by hydrophobic stacking or may form pyranoanthocyanins (Boulton, 2001, Cheynier et al., 2006, Marquez et al., 2013). Both pigmented tannins and pyranoanthocyanins are resistant to bleaching in the presence of sulphur dioxide. The formation of pigmented tannins depends on the amount of anthocyanin extracted from the grape skin and both the amount and conformation of proanthocyanidins extracted from the skin, seed and stalks of the grape.

Determining the relative amounts of phenolic compounds extracted from these tissues is largely under the control of the oenologist (Kennedy, 2008).

The phenolic composition of component berry parts and the effect of winemaking techniques on their extraction into red wine highlights differences between red grape varieties (Sacchi et al., 2005). For example, the phenolic composition of seeds has been associated with wine sensory properties such as astringency and bitterness (McRae and Kennedy, 2011, Vidal et al., 2003a, Canuti et al., 2012, Vidal et al., 2003b). Within the flavanol series, bitterness decreases and astringency increases from monomer to trimer (Noble, 1998). In their studies with red wine grapes Mattivi et al. (2009) found that in Pinot Noir grapes, 98% of flavanol monomers and 80% of oligomers came from the seeds. Until recently, most of the studies on wine pigmentation have been conducted in model solutions containing individual flavanols and anthocyanins, making it difficult to extrapolate the results to complex solutions such as red wines. This study employed a micro-vinification technique (Dambergs and Sparrow, 2011) to examine the contribution of skins and seeds of *Vitis vinifera* cv. Pinot Noir to the colour stability of red table wine.

The study compared a normal seeded PN clone with a mutant seedless clone discovered in a Tasmanian vineyard, in which the seeds abort early in the development of the grape. Earlier studies to examine the influence of seeds on red wine composition have used the addition of seed oenotannins to fermenting musts (Neves et al., 2010, Vivas et al., 2003). In this study a second experiment was undertaken to determine the effect of such additions on Pinot Noir wines.

6a.3 MATERIALS AND METHODS

Previous experiments conducted during the course of this investigation (Chapters 4 and 5), demonstrated that after fermentation, large amounts of tannin remained in seeds which are normally discarded as a component of the grape marc. As this tannin source is readily available in most wineries, this study examined the potential of fermented grape seeds to supplement the phenolic composition of Pinot Noir wines.

6a.3.1 Grape sampling and replication

Grapes of *Vitis vinifera* cv. Pinot Noir of unknown clone were used for the comparison of seeded and seedless grapes, while the experiment with oenotannin addition used Pinot Noir clone G5V15. For each experiment, grape bunches were divided into four replicates. Prior to preparing treatments for fermentation, 100 berries were selected at random to characterise grape composition of the clone. Total soluble solids in the grape juice (°Brix) were measured using a hand-held refractometer, the pH of the juice was measured using a Metrohm pH meter/autotitrator and titratable acidity was determined by titration with 0.333 N NaOH to an end point of pH 8.2 and reported as g/L tartaric acid. A further 200 g of berries from each replicate were used to assess grape colour (Iland et al., 2004) and tannin content (Dambergs et al., 2012b).

6a.3.2 Seeded vs seedless Pinot Noir

Wine from an unknown seeded clone of Pinot Noir was compared with wine made from a seedless mutant (clone unknown). Grapes were harvested from a vineyard in northern Tasmania in early in April 2011, and randomly allocated to four 1.1 kg replicates. For each replicate, 1.1 kg of grapes were manually de-stemmed, crushed and placed in a 1.5 L Bodum® coffee plunger to ferment. Treatments for this experiment were: (1) Seeded berries (control) and (2) Seedless berries.

6a.3.3 Seed Tannin Addition

To confirm effects of seed tannins, exogeneous sources of these were added to the must. Grapes for this experiment were harvested from a second vineyard in northern Tasmania in late March 2012. Initially 10 kg of Pinot Noir grapes (mixture of clones D5V12 and D4V2) were fermented to produce 200 g of fermented grape seed. A week later a further 15 kg of Pinot Noir grapes (Clone G5V15) were harvested from the same vineyard and bunches randomly allocated to 12 x 1.5 kg replicates. For each replicate, 1 kg of berries were crushed and placed in a 1.5 L Bodum® coffee plunger to ferment. The control treatment had no additional grape products. For the second treatment, a fresh stock solution of 20% (w/w) commercial grape seed tannin was prepared in de–ionised water and 2 ml added to another set of four replicates making a tannin addition of 0.4 g/L. To prepare the third treatment, 17 g (40% (w/w) of total seed from 1 kg of grapes) of fermented Pinot Noir grape seed was added to each of four replicates. Treatments for this experiment were: (1) Pinot Noir berries (PN control); (2) Pinot Noir berries plus 0.4 g/L commercial grape seed tannin (GST); (3) Pinot Noir berries plus 40% (w/w) fermented Pinot Noir seed (FPNS). Changes in phenolic composition were detected from the first day of fermentation (data not shown) however this report focuses on the phenolic composition of these wines at bottling and at 6 and 12 months bottle age (Table 6a.4).

6a.3.4 Microvinification protocol

Wine was made using micro-fermentation techniques (Dambergs and Sparrow, 2011) as described in Chapters 2, 3 and 5. After crushing, 50 mg/L SO_2 was added to each fermentation vessel in the form of potassium metabisulphite and the 'must' refrigerated overnight at 4^{o}C. The following day the grape must preparations were allowed to equilibrate at 25^{o}C, then inoculated with 300 mg/L RC212 yeast solution and incubated at

25°C (±1°C). On day 3 of the fermentation, 300 mg/L of diammonium phosphate was added to each fermentation vessel to provide 60 mg/L yeast assimilable nitrogen. After 8 days, wines were fermented to dryness which was confirmed by testing for residual sugar (< 2 g/L) using Clinitest™ reagent tablets (Bayer Australia Ltd.). The wine was hand-pressed and stored at 4°C for 14 days, at which time it was racked and a further 80 mg/L SO_2 added. The wine was stored at 12°C for a further 30 days prior to bottling. Each wine was divided into four 375 mL green glass bottles with screw caps and stored for 6 or 12 months at 12°C.

6a.3.5 Quantifying seed influence on wine phenolics

Wines were allowed to equilibrate at room temperature and clarified by centrifugation at 5,000 rpm for five minutes using an Eppendorf 5417C centrifuge with an Eppendorf F45-30-11 rotor (Crown Scientific, Australia). Incubations were performed in 10 ml centrifuge tubes, capped and inverted several times to allow for mixing. Spectrophotometric analysis was conducted using a UV-Vis Spectrophotometer (Model Genesys™ 10S Thermo Fisher Scientific Inc., Madison, WI, USA). The following assays were conducted: Assay A: One in 10 dilution of wine in buffer 1 (model wine: saturated solution of potassium hydrogen tartrate in 12% v/v ethanol) plus 0.1% v/v acetaldehyde. Samples were incubated at room temperature for 1 h. Assay B: One in 10 dilution of wine in buffer 1, plus 0.375% w/v sodium metabisulphite. Samples were incubated at room temperature for 1 h. Assay C: One in 50 dilution of wine in 1 M HCl. Samples were incubated at room temperature in the dark for 3 h. Absorbance for samples from each assay was read at 2 nm intervals from 200 to 600 nm. From these measures free anthocyanin, non-bleachable pigment, percentage non-bleachable pigment, wine hue (Mercurio et al., 2007) and rapid tannin analysis (Dambergs et al., 2012b) were calculated.

6a.3.6 Proanthocyanidin (PA) composition of wines

At 12 months bottle age, wine tannin concentration was also determined using the methyl cellulose precipitable tannin assay (MCPT) described by Sarneckis et al. (2006) and proanthocyanidin subunit composition determined using acid catalysis in the presence of excess phloroglucinol (Kassara and Kennedy, 2011). Six composition measures were calculated: percent trihydroxylated proanthocyanidin subunits (% triOH), an indicator of the proportion of skin tannin in wine; percent galloylated proanthocyanidin subunits (% gall), an indicator of the proportion of seed tannin in wine; mean degree of polymerization (mDP), a measure of the mean number of extension to terminal subunits in wine tannin polymers; mass conversion percentage (% MC), the recovery of depolymerised subunits; and molecular size at 50% elution by gel permeation chromatography (50% GPC), an indicator of the median size of tannin polymers in wine.

Tannins having a high mass conversion percentage indicate a higher proportion of depolymerised, grape-like tannins. The measures mDP, percent trihydroxylation, and percent galloylation can only be applied to proanthocyanidin subunits that have been recovered by phloroglucinolysis and are therefore interpreted relative to the mass conversion percentage.

As replicate samples of the wines were not analysed for subunit composition, only the parameters with the most profound differences have been considered in the results and discussion sections. These have been addressed in light of the highly replicable results found for the treatment wines when analysed by spectroscopy.

6a.3.7 Statistical analysis

Means and standard deviations of the free anthocyanin concentration, tannin concentration, non-bleachable pigment content, percentage non-bleachable pigment,

wine colour density and wine hue of each treatment were calculated using analysis of variance (GENSTAT 13th Edition ANOVA). The phenolic composition of each treatment that was assessed at three time intervals was interrogated using Repeated Measures ANOVA. In each case ANOVA was followed by post-hoc analysis using Fisher's Protected Least Significant Difference (LSD) test.

6a.4 RESULTS

6a.4.1 Grape composition

In addition to assessment of the composition of grapes of the variety *Vitis vinifera* Pinot Noir used to compare the seeded and seedless clone, the composition of Pinot Noir clone G5 V15, used in the 'added seed tannin' experiment was also evaluated (Table 6a.1). Berries of the seedless clone were much smaller than those of the seeded clone, with diameter 33% that of the seeded clone and volume 10% that of the seeded clone. While the total soluble solids content of the seedless clone was higher than the seeded clones, the total phenolic concentration of berries was similar for each clone (1.0 ± 0.1 AU/g).

The total tannin concentration of the seeded clones was double that of the seedless clone, whereas the anthocyanin concentration of the seedless clone was three times higher than the seeded clones.

Table 6a.1: Grape composition (mean and standard deviation, n=3) of seeded and seedless clones of *Vitis vinfera* cv. Pinot Noir

Berry composition	Seeded clone	Seedless clone	Clone G5V15
Berry Wt (g FW‡)	1.33 ± 0.03	0.44 ± 0.01	1.15 ± 0.03
Sugar (°Brix)	21.5 ± 1.41	29.4 ± 1.13	22.6 ± 0.28
pH	3.12 ± 0.04	3.31 ± 0.02	3.13 ± 0.04
Titratable acidity (g/L)	11.1 ± 1.17	12.8 ± 0.72	9.50 ± 0.17
Anthocyanin (mg/g FW‡)	0.63 ± 0.06	1.98 ± 0.07	0.68 ± 0.01
Total tannin (mg/g FW‡)	5.87 ± 0.49	2.99 ± 0.22	6.84 ± 0.30
Total phenolics (AU/g)	1.03 ± 0.05	1.09 ± 0.04	1.23 ± 0.05

‡FW, fresh weight

6a.4.2 Changes in wine phenolic composition from bottling to 12 months bottle age

Seeded versus seedless clones:

The free anthocyanin content of wine made from the seeded clone declined 0.31 g/L to 0.20 g/L (30%, $P<0.001$) and from 1 g/L to 0.59 g/L (39%, $P<0.001$)in wine from the seedless clone between bottling to 12 months bottle age. The difference in magnitude of anthocyanin in the two clones reflects the difference in the surface to volume ratio of the berries, whereas the tannin content of both clones remained stable over this time period (Table 6a.2). The non-bleachable pigment concentration increased by 30% ($P<0.001$) in the seeded clone and 40% ($P<0.001$) in the seedless clone during 12 months in the bottle, while at the same bottle age, the percentage of the pigment that was non-bleachable relative to total pigment was 4.4% ($P<0.001$) in the seeded clone and 2.5-fold higher (10.8%; $P<0.001$) in the seedless clone. No

significant change in colour density was observed in wine made from either clone (data not shown) but there was a major decline in wine hue (25-fold, $P<0.001$) in wines made from the seedless clone as they aged (more purple tones) and a lesser (5-fold, $P<0.001$) decrease in wine hue in seeded wines (Table 6a.2). The proportion of pigments that were non-bleachable and the low hue indicated the development of stable colour pigments in 12 month old wines.

Table 6a.2 Phenolic characteristics of wine made from seeded and seedless clones of Pinot Noir at bottling, 6 and 12 months bottle age. Data are Mean ± Standard Deviation (n=4) of each phenolic parameter

		Bottle age		
Phenolic character	Bottling	6 Months	12 Months	***P-value***
Anthocyanin (g/L)				
Seeded clone	0.31 ± 0.01	0.26 ± 0.01	0.20 ± 0.01	*<.001*
Seedless clone	1.00 ± 0.03	0.82 ± 0.03	0.59 ± 0.04	
Tannin (g/L)				
Seeded clone	0.66 ± 0.03	0.65 ± 0.03	0.67 ± 0.04	*0.617*
Seedless clone	2.03 ± 0.21	2.03 ± 0.19	2.06 ± 0.23	
Non-bleachable pigment (AU)[‡]				
Seeded clone	0.31 ± 0.04	0.37 ± 0.03	0.48 ± 0.04	*<.001*
Seedless clone	1.77 ± 0.25	2.76 ± 0.40	3.87 ± 0.48	
% non-bleachable pigment				
Seeded clone	1.92 ± 0.19	2.70 ± 0.16	4.40 ± 0.27	*<.001*
Seedless clone	3.33 ± 0.33	6.03 ± 0.57	10.72 ± 0.55	
Hue (AU)[†]				
Seeded clone	0.57 ± 0.01	0.69 ± 0.00	0.73 ± 0.00	*<.001*
Seedless clone	0.55 ± 0.02	0.62 ± 0.01	0.63 ± 0.01	

[‡]Non-bleachable pigment calculated from the absorbance at 520 nm multiplied by 10, of the wine sample diluted 1:10 in model wine solution containing 0.375% sodium metabisulphite. [†]Hue calculated from the ratio of absorbance of at 420 nm /520 nm for wine samples diluted 1:10 in model wine solution.

In addition to the determination of wine phenolic parameters by spectral analysis, the Methyl Cellulose Precipitable Tannin (MCPT) content of each wine (mg/L) was determined at 12 months bottle age. The MCPT concentration of the seedless clones of Pinot Noir was found to be 3-fold higher than the seeded clone, whereas the mean degree of polymerisation (mDP) was apparentlylower for the seeded clone (5.7 mDP) than for the seedless clone (7.3 mDP) (Table 6a.3).

Table 6a.3: Proanthocyanidin (PA) subunit composition of wine at 12 months bottle age made from seeded and seedless clones of Pinot Noir

Clone	MCPT[†]	mDP[‡]	% MC[§]	% tri-OH[φ]	% gall[Ƀ]	tri-OH/gall[€]	50% GPC[¥]
Seeded	0.54	5.7	88.1	20.0	2.9	6.9	1584
Seedless	1.51	7.3	49.1	21.7	3.2	6.8	1541

[†]Methyl Cellulose Precipitable Tannin (g/L berries); [‡]Mean degree of polymerization (mDP) based on subunit composition from phloroglucinolysis; [§]%MC, Mass conversion percentage; recovery of PA subunits by phloroglucinolysis based on the gravimetric mass; [ϕ]% tri-OH, percentage trihydroxylated PA subunits; [Ƀ]% gall, percentage galloylated PA subunits; [€] tri-OH/gall, ratio of trihydroxylated PA subunits to galloylated PA subunits; [¥]50% GPC median molecular size assessed by Gel Permeation Chromatography (g/mol). Duplicate samples of each treatment were analysed.

The mass conversion percentage (representing the proportion of the tannin that can be depolymerised by the assay) was 1.8 fold higher for the seeded clone than for the seedless clone. The ratio of trihydroxylated to galloylated proanthocyanidin subunits was similar for each clone (Table 6a.3). As trihydroxylated subunits do not occur in seeds, the recovery of trihydroxylated subunits by phloroglucinolysis is an indication of proanthocyanidins derived from skin that have not become polymerised and was found to be similar for each clone.

This indicated that a greater proportion of proanthocyanidin polymers were formed from galloylated subunits than from trihydroxylated subunits. he large difference in mass conversion percentage between wines from each clone, suggested that a much greater proportion of stable proanthocyanidin polymers were formed from skin tannins in wines made from the seedless clone than wines made from the seeded clone. Taken together, these results point to the possibility of proanthocyanidins which are rich in galloylated subunits being less likely to polymerise or more easily depolymerised than are trihydroxylated subunits.

Seed Tannin addition:

Phenolic composition of the fermenting wine was analysed from the first day of fermentation, however the reported results focus on the phenolic composition of the wines analysed at bottling, six and 12 months bottle age. As there was no interaction between time and treatment, the value of the time effect shown for each parameter in Figure 6a.4 is the average across the three treatments while the value of the treatment effect is the average across the three time periods(Table 6a.4). Full details for each treatment effect at each time period is listed in Appendix 6a.1.

The tannin content of wine with added grape seed tannin (GST) was 47% (P=0.003) higher than the control wine, while the addition of fermented Pinot Noir seeds (FPNS) resulted in a 90% (P=0.003) increase in tannins (Table 6a.4). The magnitude of the difference between the control and treatment wines was similar at 6 and 12 months bottle age (P=0.012). While the free anthocyanin content of each wine declined during 12 months of bottle ageing, the anthocyanin content of the exogenous tannin treated wines remained 43% (P<0.001) higher than the control. The non-bleachable pigment content of control wines increased 3-fold (P<0.001) from bottling through 12 months of age, whereas when either

source of exogenous seed tannin was added, the increase in non-bleachable pigment was only 2-fold (P<0.001). Wine colour density of the control wine increased by 63% (P=0.008) from bottling to 12 months bottle age, but was significantly less (25% increase; P=0.008) in treatments with added seed tannin. The value of wine hue in the presence of sulphur dioxide gave an indication of the relative amounts of stable red to purple colouration in the wine.

Table 6a.4 Changes in the phenolic characteristics of Pinot Noir wines at bottling, six and 12 months bottle age (mean n=4). Comparison of must with no addition to must with additions of grape seed tannin (GST) or fermented Pinot Noir seeds (FPNS).

	Bottle age (Months)				Wine with added seed tannin			
Phenolic character	0	6	12	$^{₮}LSD_{0.05}$ *(time)*	None (control)	GST (4g/L)	FPNS (17g/L)	$^{€}LSD_{0.05}$ *(trt)*
anthocyanin (mg/L)	324 c	236 b	188 a	*23.23*	191 a	291 b	266 b	*23.7*
tannin (g/L)	1.05 a	1.05 a	1.15 b	*0.07*	0.74 a	1.09 b	1.41 c	*0.27*
NB pigment (AU)	0.56 a	1.24 b	1.46 b	*0.47*	1.31	0.90	1.04	*NS*
colour density	4.46 a	6.10 b	6.03 b	*0.99*	5.77	5.26	5.56	*NS*
total phenolics (AU)	44.41	43.0	43.75	*NS*	36.5 a	44.7 b	49.9 b	*5.43*
hue SO_2 (AU)	1.56 b	1.13 a	1.11 a	*0.06*	1.18 a	1.32 b	1.30 b	*0.09*

$^{₮}$LSD, Least Significant Difference over time, using repeated measures ANOVA; $^{€}$LSD, Least Significant Difference between treatments, using one way ANOVA. For each statistical analysis, phenolic characters values with different letters are significantly different at P ≤ 0.05. NS, Not significant at P ≤ 0.05.

The Hue SO_2 value decreased by 48% (P<0.001) from bottling to 12 months bottle age in control wines indicating the development of more blue-purple tones in the wine, whereas the decrease (33%; P<0.001) was significantly less in wines treated with additional seed tannin. The differences in anthocyanin, non-bleachable pigment and hue determined by spectral analysis confirmed visual observations that wines with additional seed tannin appeared to be significantly more red and less purple in colour than control wines at 12 months bottle age.

6a.5 DISCUSSION

The loss of colour in red wines during the months following fermentation is a familiar one that has been widely reported and comprehensively reviewed (Boulton, 2001). The formation of stable colour pigments by the development of specific species of pyranoanthocyanins and the polymerisation of anthocyanins with tannins helps to reduce colour loss as the wine ages. This study demonstrated the critical importance of the presence of skin versus seed derived tannin in determining the colour stability of Pinot Noir wines. Tannins with a higher degree of polymerisation are generally more stable than tannins with lower mDP (Boulton, 2001, Cheynier et al., 2006, Ribereau-Gayon et al., 2006, Mattivi et al., 2009, McRae and Kennedy, 2011). Maceration techniques that involve longer extraction times before and after fermentation aim to increase the stable pigment content of the wine (Sacchi et al., 2005) but unless over-extraction of seed tannins is avoided, this investigation demonstrated that such an outcome cannot be assured.

6a.5.1 Wine made from seeded vs seedless grapes

The advent of the seedless clone was very beneficial to this investigation on the nature of skin versus seed tannin in Pinot Noir. The small size of berries of the seedless clone arises

as a hormonal response to the absence of seeds resulting in poor development of the fleshy endocarp. The bunch size was not effected by the lack of seeds. The difference in the surface to volume ratio of the berries, accounts for significant differences in anthocyanin concentration of the wines, while the difference in tannin concentration gives greater insight into the extraction of skin tannin and the development of non-bleachable pigment that can take place when seed tannin is not involved. The higher percentage of non-bleachable pigment detected in the seedless clone at each time period confirms that tannins derived from grape skin form more stable pigmented tannins than do tannins derived from seeds (Boulton, 2001), which was also confirmed by experiments reported in Chapters 4 and 5. This observation was supported by the lower mass conversion percentage for wine made from seedless grapes, which indicated that approximately half (51%) of the tannin in the wine made from seedless grapes had become polymerised in the first 12 months of bottle ageing (McRae et al., 2010, Poncet-Legrand et al., 2010, McRae and Kennedy, 2011) and was not depolymerised by acid-catalysis in the presence of phoroglucinol. By comparison the proportion tannin that could be depolymerised by phloroglucinolysis in the seeded clone was relatively high. The mass conversion percentage of 88% implied that only 12% of polymerised tannins could not revert to proanthocyadin subunits following phloroglucinolysis (Table 6a.3). As trihydroxylated tannins do not occur in grape seeds (Prieur et al., 1994, Souquet et al., 1996, Hayasaka et al., 2003, McRae and Kennedy, 2011), the ratio of trihydroxylated subunits to galloylated subunits gives an indication of the source of the tannin in the wine, assuming that the depolymerisation reaction is non-selective. In comparing the seeded and seedless clone, the ratio of trihydroxylated to galloylated subunits was similar (Table 6a.3), indicating that of the tannin that could be depolymerised, a similar proportion in each clone was skin

derived. Proanthocyanin polymers with a high degree of galloylation tend to form simple chains rather than their more compact counterparts containing trihydroxylated subunits due to stereochemical restrictions imposed by the gallate moeity attached to the B-ring of the proanthocyanidin subunit (Scollary, 2010). The lower concentration of galloylated tannins available for polymerisation in the seedless clone may explain both the lower mass conversion percentage observed for these wines and the similarity in the ratio of trihydroxylated to galloylated subunits for wines from each clone (Table 6a.3) Quite surprisingly, a preliminary tasting conducted by three experienced tasters found that while the colour intensity, fruit and floral flavours of the wine made from the seedless clone were significantly greater than for wine made from the seeded clone, the seedless wines were clearly less astringent, subsequently compromising the palate length.

6a.5.2 Wine made with seed-derived tannin additions:

Commercial grape seed tannin (GST) is composed of condensed tannin (oligomeric proanthocyanidin and proanthocyanidins) extracted from white grape pomace (Tarac Technologies, 2012). In this experiment, the control wines at 12 months bottle age had a higher proportion of non-bleachable pigments and lower hue than the wines treated with exogenous seed tannin, that may have been formed by the development of pyranoanthocyanins (Boulton, 2001, Cheynier et al., 2006, Zanchi et al., 2007). Meanwhile the wines with added seed tannin had a high hue value that might be attributed to the presence of free anthocyanins (Ribereau-Gayon et al., 2006) or pigmented tannins (Somers and Evans, 1977). The results suggest that components of the seed-derived tannin caused the anthocyanin to remain free in the wine matrix. Although the total tannin content of the wine in the control treatment remained the same from bottling to 12 months bottle age, the addition of 0.4 g/L GST to the must prior to fermentation, was found to increase

the total tannin content of the wine by a corresponding 0.4 g/L. As the addition of 17 g of fermented seed was shown to increase the total tannin content of the wine by 0.7 g/L, the amount of tannin retained in the fermented Pinot Noir seed was calculated at 40 mg/g of seed or 1.3% of the total grape tannin (Table 6a.1), equivalent to 97% of the wine tannin (Table 6a.4). This finding has significant implications for winemaking processes that involve the addition of commercially available tannin supplements or extended maceration processes which do not distinguish between tannin sources. Such techniques may inadvertently increase the seed tannin content of the wine, thereby compromising the development of stable pigmented tannins and compromising wine colour stability (Boulton, 2001, González-Manzano et al., 2004, Ribereau-Gayon et al., 2006).

The observations made on Pinot Noir wine tannins described in this study add to the current understanding of this unique variety. In their work with Cabernet Sauvignon, Rolle et al. (2012), quoting the work of Gonzàlez -Manzano et al. (2012) and Cheynier et al. (2006), stated that 'seeds are rich in flavan-3-ols, which are better anthocyanin co-pigments than flavanols found in skins and therefore contribute to long term colour stability'. By contrast, we found that when sources of seed tannin were added in excess of quantities inherent in Pinot Noir grapes, the formation of stable pigments was compromised. In their review of wine pigments and tannins, Cheynier et al. (2006) acknowledged the very simple anthocyanin composition of Pinot Noir, being a cultivar that has no acylated or coumarylated anthocyanins, making it unlike most other grape varieties (Mazza et al., 1999, Heazlewood et al., 2006, He et al., 2010, Ferrandino et al., 2012). In comparing a range of red wine varieties Mattivi et al. (2009) reported that while the percentage of epicatechin gallate proanthocyanidin subunits was similar in Pinot Noir, Cabernet Sauvignon, Merlot and Syrah grapes, Pinot Noir grapes were 3 to 4 times higher

in proanthocyanidin monomers and 2 to 3 times higher in oligomers than the other red grape varieties. They also reported that the quantity of seed oligomers was 4.3-fold higher than skin oligomers. As monomers and oligomers have both been associated with bitterness (Noble, 1998), a higher proportion of these proanthocyanidins in the wine may have significant bearing on the sensory attributes of Pinot Noir.

Di Stefano et al. (1990) reported that the galloylated proanthocyanidins from seeds are a source of free gallic acid in the wine. In subsequent work with Castelão and Tinta Miúda varieties, Neves et al. (2010) showed that the addition of exogenous grape seed tannins increased the colour intensity and antioxidant activity of wines that were known to be poor in polyphenols. In addition they found that more gallic acid was released into the wine matrix when grape seed tannins were added, even though no gallic acid was detectable in the tannin additive itself.

Considering this observation by Neves et al. (2010) and the experiments reported in this study, one might propose that gallic acid was released from seeds either directly or was the product of polymerisation reactions of galloylated tannin subunits. Either process would confer a higher concentration of gallic acid to the wine matrix, potentially reducing the pH (Brouillard and Dangles, 1994, Zanchi et al., 2007, Ferrandino et al., 2012). Such an explanation would account for the predominantly reddish colour observed in young Pinot Noir wines. Meanwhile, the higher proportion of proanthocyanidin monomers and oligomers relative to larger polymers that have been reported to occur in Pinot Noir wines (Mattivi et al., 2009) could limit the protection of the anthocyanin moiety from oxidation (Boulton, 2001, Cheynier et al., 2006, Ribereau-Gayon et al., 2006, McRae and Kennedy, 2011). This would make Pinot Noir wines more susceptible to tawny discolouration as they age.

6a.6 CONCLUSION

The investigation suggested that wine made from *Vitis vinifera* cv. Pinot Noir behaves differently to other red wine varieties in the first 12 months after bottling. Yet the absence of seed derivatives in the seedless clone allowed the formation of pigmented polymers formed from skins only to be viewed more objectively. When seeds were absent, the formation of pigmented polymers continued at the same rate from bottling to 12 months bottle age, whereas in the presence of seeds the formation of stable pigments was significantly slower over the same time period. Tannin polymers formed in the presence of seed derivatives were smaller and less stable than those formed from skin derivatives only. The addition of exogenous seed tannin increased both the tannin composition and brightness of the wine. A probable explanation is that the nature of the pigments, either as free anthocyanins or pigments polymerised with seed derived proanthocyanidins, resulted in entities that were less likely to form large stable pigmented polymers, potentially limiting the colour stability of the wine.

In conclusion, it is likely that maceration techniques that target the extraction of skin tannins from Pinot Noir grapes, while simultaneously limiting seed tannin extraction, are likely to be most beneficial for the optimising the phenolic composition of Pinot Noir wines.

Appendix 6a.1 Changes in the phenolic characteristics of Pinot Noir wines at bottling, six and 12 months bottle age (Mean n=4). Comparison of must with no addition (control) to must with additions of grape seed tannin (GST) or fermented Pinot Noir seeds (FPNS).

Treatment	Bottle Age			
	Bottling	6 months	12 months	*P-value (time)*
		Anthocyanin (mg/L)		
PN control	272 b	157 b	146 c	
PN + GST	356 a	285 a	232 a	*<0.001*
PN + FPNS	343 a	268 a	185 b	
		Tannin(g/L)		
PN control	0.69	0.72 ab	0.81 b	
PN + GST	1.08	1.05 b	1.14 b	*0.012*
PN + FPNS	1.37	1.37 a	1.49 a	
		NB pigment		
PN control	0.56 a	1.63 a	1.73 a	
PN + GST	0.54 a	1.02 b	1.16 b	*<0.001*
PN + FPNS	0.57 a	1.07 b	1.48 ab	
		Colour Density		
PN control	4.13	6.73	6.45	
PN + GST	4.55	5.73	5.50	*0.008*
PN + FPNS	4.68	5.84	6.14	
		Total Phenolics (AU)		
PN control	37.0 b	35.8 b	36.8 b	
PN + GST	45.6 a	44.0 a	44.6 a	*0.003*
PN + FPNS	50.6 a	49.3 a	49.8 a	
		Hue SO_2		
PN control	1.52	0.99 b	1.03 b	
PN + GST	1.57	1.20 a	1.18 a	*<0.001*
PN + FPNS	1.58	1.19 a	1.13 a	

Data are treatment means at each time period (n=4); For each treatment, numbers within a column followed by different letters indicate significant differences at $p \leq 0.05$ at the specified time period.

6a.7 SUBSEQUENT RESEARCH FOCUS

Having confirmed that excess seed tannins or their derivatives may have detrimental effects on the colour stability of Pinot Noir wines, complementary ferments were examined where a proportion of seeds were removed during fermentation. In recognition of the practical application of seed removal to a commercial winery situation, only the seeds that had fallen to the bottom of the fermentation vessel were sequentially removed. The histochemical features of the removed seeds and the phenolic composition of the resulting wine are described in Chapter 6b.

6B EFFECT OF SEED REMOVAL DURING FERMENTATION ON THE PHENOLIC COMPOSITION OF PINOT NOIR WINE

This chapter is in preparation as a research journal article.

Title: **'***Seed Removal during Fermentation Improves the Phenolic Quality of Pinot Noir Wine'*

Authors: *Angela M. Sparrow, Warwick Gill, Robert G. Dambergs and Dugald C. Close*

6b.1 ABSTRACT

Colour pigments and tannin are the two major classes of phenolics important to red winemaking, both of which are found in grape skins. However, in *Vitis vinifera* cv. Pinot Noir grapes, the concentration of tannin is much greater in the seeds than in the skins. This study examined the phenolic characteristics of wine made with altered seed contact time during fermentation using submerged cap microvinification techniques. Seeds that had been released from the pomace cap during the fermentation were removed from the fermentation vessel on either day 1, 3 or 5 of fermentation and the location of flavan-3-ols within these seeds assessed by histochemistry. The diffusion of tannin from the outer integument towards the epidermis of the seed commenced within 24 hours of yeast inoculation, however the majority of seed tannin was not released into the wine matrix until day 6 of fermentaion. The phenolic characteristics of the wines at six months bottle age were estimated using utraviolet spectroscopy and chemometrics. The impact of removing only those seeds that had been released from the floating pomace cap of the ferment, was found to modify the tannin concentration of the wines. Within 24 hours of inoculation, 15% of seeds were released from the pomace cap, and their removal coincided with a 14% reduction in the concentration of wine tannin. When removal of seeds was delayed until day 3 of fermentation, 26% of seeds had been released and by the fifth day, 35% of the total number of seeds in the ferment were released and could be removed from the ferment.

The inclusion of pectolytic enzyme in the ferments made little difference to the number of seeds released but caused the tannin concentration of the wines to increase by 49%. Absorbance of the wine at 220 nm was strongly correlated with the tannin concentration of the wine at six months bottle age (r^2 =0.99) and was attributed to the presence of proanthocyanidin subunits and to gallic acid. These findings have implications for differentiating seed and skin phenolic derivatives by spectroscopy.

6b.2 INTRODUCTION

Grape berries are composed of three major tissue types: seed, skin, and pulp. In red winemaking the skins are of primary importance, as they contain two main classes of polyphenols: the colour pigments (anthocyanins) and proanthocyandins (condensed tannins or 'tannins') which are responsible for colour density, stability and mouth feel properties of the wine (Canuti et al., 2012, McRae and Kennedy, 2011, Vidal et al., 2003a). While seeds represent less than 7% of the berry weight, they are a large source of phenolic compounds, as recognised in previous research (Sun et al., 1999, González-Manzano et al., 2004) and in experiments reported in Chapter 4. Even though seed tannins are less readily extracted than are skin tannins (Ribereau-Gayon et al., 2000) they are especially important in red wine varieties that have unusual polyphenolic profiles such as Pinot Noir, Nebbiolo, Gamay, Sangiovese and Barbera**.** In the case of Pinot Noir, earlier studies have shown that the ratio of seed to skin tannin in Pinot Noir grapes is skewed in favour of seed tannins by approximately 3:1 (Cortell et al., 2005, Cortell and Kennedy, 2006, Cortell et al., 2007, Kemp et al., 2011).

Several studies on the influence of seed tannin in red wine making have focused on the addition of seed tannin extracts to wines that are poor in polyphenols, with the aim of

acheiving a more robust wine (Kovac et al., 1995, Vivas et al., 2003, Neves et al., 2010). However in experiments described previously (Chapters 4, 5 and 6a), it was demonstrated that the addition of exogenous seed tannins to Pinot Noir musts may have a detrimental effect on wine colour stability, high concentration of free anthocyanins indicating that both the association of anthocyanin molecules with one another and the formation of pigmented tannins may have been restricted. In addition to compromising the wine colour, seed tannins have also been associated with bitter flavours (McRae and Kennedy, 2011, Vidal et al., 2003a, Canuti et al., 2012).

In contrast to the studies where seed tannins have been added to red wine ferments, fewer studies have investigated the removal of whole seeds. A report by Lee et al. (2008) described the removal of seeds during periodic pump-overs during vinification of Merlot wines in a commercial winery, but found differences in the tannin composition of the wine to be minor.

Changes to the phenolic characteristics of wine caused by different amounts of seed contact time during fermentation, were assessed by progressively removing a proportion of seeds during fermentation. Seeds were removed either one, three or five days after inoculation of an eight-day ferment. The mobilisation and release of tannin from the seed tissues was monitored by histochemistry.

Research on the ability of pectolytic enzymes to enhance phenolic extraction in red wines has been extensively reported (Pardo et al., 1999, Bautista-Ortín et al., 2005, Ducasse et al., 2010b, Sacchi et al., 2005, Meyer et al., 1998), consequently the impact of pectolytic enzymes on the release of seeds from the pomace was also investigated.

6b.3 MATERIALS AND METHODS

6b.3.1 Grape sampling and analysis

Grapes of *Vitis vinifera* cv. Pinot Noir clone G5V15, from drip irrigated vines, trained to vertical shoot positioning, were harvested from a 14 year old vineyard in northern Tasmania in April 2012. Fruit was hand harvested and bunches were randomly allocated to four 2.5 kg replicates. Prior to fermentation, 100 berries were selected at random to determine grape composition. The berries were hand-crushed and the juice drained for grape composition analyses. Total soluble solids (°Brix) were measured using a hand-held refractometer, the pH of the juice was measured using a Metrohm pH meter/autotitrator and titratable acidity was determined by titration with 0.333 M NaOH to an end point of pH 8.2 and reported as g/L tartaric acid. A further 200 g of berries were frozen at 20 ^{o}C for later analysis of grape colour (Iland et al., 2004) and tannin (Dambergs et al., 2012b).

6b.3.2 Microvinification treatments

Wine made from must fermented according to a standard submerged cap microvinification procedure (Sparrow et al., 2012b, Smart et al., 2012, Sparrow et al., 2012a) was compared with wines from which the seeds were removed on separate days of fermentation. For each replicate, grapes were removed from the stalks by hand and divided into four groups each weighing 200 g. The grapes were crushed by hand and placed in 250 mL fermentation vessels. At the same time of day on the day specified by the treatment, seeds that had been released from the pomace cap and fallen to the base of the fermentation vessel were removed from the ferment. Treatments for this trial were: (1) Berries (control); (2) Seeds removed day 1; (3) Seeds removed day 3; (4) Seeds removed day 5.

In order to assess the influence of pectolytic enzyme on berry tissue breakdown and subsequent rate of seed release, the four treatments were repeated with 300 mg/L Lafase HE (Lafazyme) pectolytic enzyme added to the must two hours prior to inoculation.

6b.3.3 Seed removal:

Seed removal was implemented by removing from the fermenting wine the spacer and gauze mat used to effect submerged cap micro-vinification allowing the pomace cap to re-float. After 1 hour, the pomace cap was removed from the fermenting wine using a gauze sieve (2 mm pore size) and transferred to a separate vessel. The fermenting juice that remained was passed through a second gauze sieve to collect the seeds which had fallen to the bottom of the fermentation vessel. After being counted these seeds were stored at 4^{o}C for histochemical analysis. The pomace cap including its suspended seeds, was then replaced and the fermentation resumed. The seeds remaining in the marc at pressing were also counted, to determine the percentage of seeds that had been released from the pomace cap and removed from the ferment on day one, three or five of fermentation.

6b.3.4 Microvinification protocol

Wine was made using micro-fermentation techniques and ferments conducted in polycarbonate jars of 250 mL capacity as described in Chapter 2 (Smart et al., 2012, Sparrow et al., 2012a, Sparrow et al., 2012b, Sparrow et al., 2013a). After crushing, 50 mg/L SO_2 was added to each fermentation vessel in the form of potassium metabisulphite and the 'must' refrigerated overnight at 4^{o}C. The following day the grape must preparations were allowed to equilibrate at 25^{o}C, then inoculated with 300 mg/L RC212 yeast solution and the ferments incubated at 25°C (±1°C). On day three of the fermentation 300 mg/L of diammonium phosphate was added to each ferment. The conclusion of fermentation was confirmed by testing after eight days with Clinitest™

reagent tablets (Bayer Australia Ltd.); all wines were found to be dry (< 2 g/L residual sugar). The wine was hand-pressed and stored at 4°C for 14 days, at which time it was racked and a further 80 mg/L SO_2 added. The wine was stored at 12°C a further 30 days prior to bottling, when each wine was decanted into two 25 mL amber glass bottles with screw caps. The bottled wine was stored for 6 months at 12°C.

6b.3.5 Quantifying the effect of seed removal on wine phenolics

The phenolic composition of the finished wines was determined using a modification of the wine colour and tannin assays described by Mercurio et al. (2007) and (Dambergs et al., 2012b).

Wine samples were allowed to equilibrate at room temperature and clarified by centrifugation at 5,000 rpm for five minutes using an Eppendorf 5417C centrifuge with an Eppendorf F45-30-11 rotor (Crown Scientific, Australia). Incubations were performed in 10 mL centrifuge tubes capped and inverted several times to allow for mixing prior to incubation. Ultraviolet-Visible spectrophotometric analysis was conducted using a UV-Vis Spectrophotometer (Model Genesys™ 10S Thermo Fisher Scientific Inc., Madison, WI, USA). The following assays were conducted: Assay A: One in 10 dilution of wine in buffer 1 (model wine: saturated solution of potassium hydrogen tartrate in 12% v/v ethanol) plus 0.1% v/v acetaldehyde. Samples were mixed and incubated at room temperature for 1 h. Assay B: One in 10 dilution of wine in buffer 1, plus 0.375% w/v sodium metabisulphite. Samples were mixed and incubated at room temperature for 1 h. Assay C: One in 50 dilution of wine in 1 M HCl. Samples were mixed and incubated at room temperature in the dark for 3 h. For each assay absorbance was read at 2 nm intervals from 200 to 600 nm. From the above measures free anthocyanin, non-bleachable pigment, wine colour density, wine hue and

hue resistant to sulphur dioxide bleaching (Mercurio et al., 2007) and total tannin concentration (Dambergs et al., 2012b) were calculated.

6b.3.6 Statistical analysis

Mean, standard deviation and co-efficient of variation were calculated for grape composition parameters. Statistical analyses were conducted using GenStat 64-bit Release 16.1 Copyright 2013, VSN International Ltd. Data for individual phenolic components in wine samples were analysed using two-way ANOVA, with day of seed removal as the main plot and enzyme addition as the sub-plot. For each of the phenolic parameters assessed, ANOVA was followed by post-hoc analysis using Fisher's Protected Least Significant Difference (LSD) test.

6b.3.7 Microscopic analysis

Free-hand transverse seed sections, approximately 1 mm thick, were fixed under vacuum in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 2 h and then for a further 12 h at 4°C. Following two buffer washes, samples were dehydrated in an ascending ethanol series in 20% increments, embedded in Technovit 7100 resin (ProSciTech, Kirwan, Qld, Australia) and polymerised for 12 h at 25°C. Semi-thick sections (4 to 6 μm) were cut with glass knives on a Reichert OmU2 ultramicrotome (New York, USA), floated onto a drop of distilled water on a glass slide and allowed to air dry.

Two staining solutions were used for each seed sample: toluidine blue O, a metachromatic reagent, was used to determine the seed structure. The different structures of grape seed tissues were coloured in shades of blue (Trump et al., 1961). Slides were immersed in 1% (w/v) toluidine blue O in 0.1 M acetate buffer for 30 seconds, rinsed in distilled water, decolourised in 70% ethanol for 30 seconds, rinsed in distilled water and air dried. The sections were mounted in Euparal (Australian Entomological Supplies, Sydney, Australia)

beneath a coverslip. Phenolic material showed as dark green-brown cellular inclusions (Feder and O'Brien, 1968, Cadot et al., 2011).

Vanillin-HCl was used to identify the catechins and condensed tannins according to Dai et al. (1995). Slides were immersed in a 10% vanillin (w/v) in a solution of 50% absolute ethanol in concentrated HCl (v/v) solution for 30 minutes, then decolourized in 95% ethanol and air dried. The sections were mounted in Euparal beneath a coverslip. Both treatments were examined with a Leica DMLB30T microscope (Leica Microsystems, Germany) fitted with standard brightfield optics. Images were captured with a Leica DFL420 camera (Leica Microsystems, Germany) and processed with Leica Application Suite version 3.6.0 software.

6b.4 RESULTS

6b.4.1 Grape composition

The composition of grapes of variety *Vitis vinifera* Pinot Noir Clone G5 V15 used in this trial are described in Table 6b.1. For each grape characteristic the coefficient of variation was less than 4.5%. The tannin and anthocyanin concentration for the grape samples were within the range reported in literature for Pinot Noir (Cortell and Kennedy 2006; Cortell et al. 2005; Cortell et al. 2007; Kemp et al., 2011).

Table 6b.1 Composition of *Vitis vinfera* cv. Pinot Noir grapes, Clone G5V15

Data are neans ± standard deviation and coefficients of variation (%CV), n=4.

Berry composition	Clone G5V15	%CV
Berry Wt (g FW[‡])	1.15 ± 0.03	2.6
Sugar (^{o}Brix)	22.6 ± 0.28	1.2
pH	3.13 ± 0.04	1.3
Titratable acidity (g/L)	9.50 ± 0.17	1.8
Anthocyanin (mg/g FW[‡])	0.68 ± 0.01	1.5
Total tannin (mg/g FW[‡])	6.84 ± 0 .30	4.4
Total phenolics (AU/g)	1.23 ± 0.05	4.1

[‡]FW, fresh weight

6b.4.2 Seed removal

The quantity of seeds that could be readily removed from the fermenting wine was assessed at two-day intervals during an eight day fermentation period (Figure 6b.1). After 24 hours of fermentation, 15% of seeds had been released from the pomace cap. When left until day 3 of fermentation, 26% of seeds were removed and when left until day 5 of fermention, 35% of the seeds were removed. The number of seeds released from the pomace cap was fewer than expected, and may be a function of submerged cap vinification, in which the pomace cap is not agitiated during fermentation, compared with traditional punch-down vinification methods. However even the removal of 15% of the seeds had a signficant impact on the phenolic concentration of the wine (Table 6b.2). The addition of pectolytic enzyme was shown to have no significant effect on the number of seeds released from the pomace cap on either day 1 or day 5 of fermentation, however on day 3 fewer seeds (19% of total seeds compared with 26% with no enzyme addition; $P<0.001$) were released. Contrary to

expections, the extent of degradation of pulp and skin cell wall material in the presence of pectolytic enzyme appears to have resulted in a greater number of seeds being retained in the pomace cap on day 3 of fermentation relative to the treatment with no pectolytic enzyme.

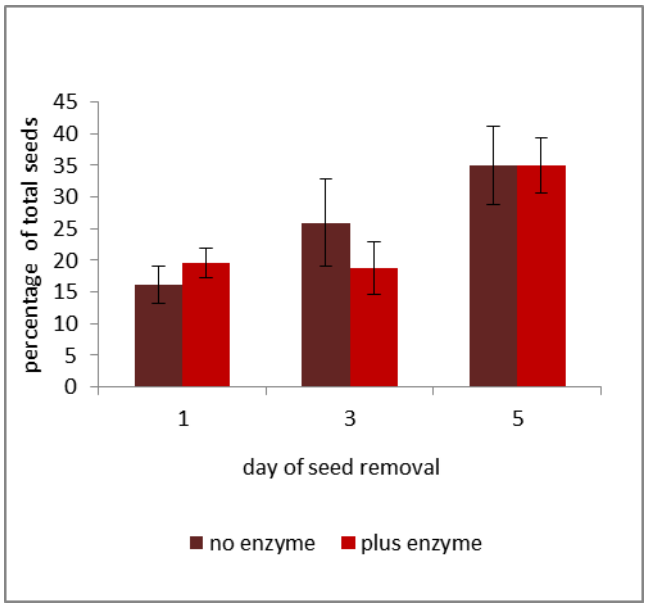

Figure 6b.1. Percentage of seeds removed from ferments on alternate days during fermentation. Data are treatment means ± standard deviation (n=3)

6b.4.3 Histochemical results

Seed tissue slices were taken in transverse-section from seeds isolated from fresh berry tissues at crushing. Staining with toluidine blue O allowed the cell types to be differentiated and identified in seeds prior to fermentation (Figure 6b.2). This image was used as a reference to identify tannins in tissues of seeds that were sequentially removed throughout the fermentation, dissected in transverse section and treated with Vanillin-HCl to stain flavan-3-ols in the cells brown (Figure 6b.3).

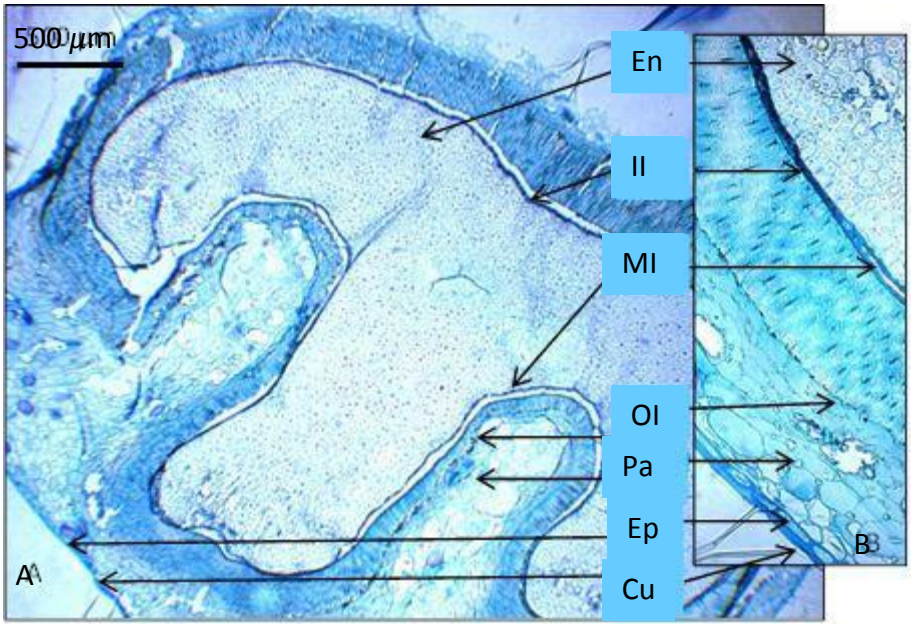

Figure 6b.2. Light micrograph transverse-section of cells of grape seeds at crushing

Five tissues were stained with Toluidine Blue O: the cuticle (Cu) epidermis (Ep); cells in the outer integument (OI); the middle integument (MI) and the last cell layer in the inner integument (II). En = endosperm; Pa = parenchyma cells in the internal zone. Panel A: 40x magnification; Panel B insert is 100x magnification of the same tissues.

As flavan-3-ols are subunits of condensed tannins, more intensely coloured cell tissues reflected a higher concentration of tannins in the cells. The greatest concentration of tannin was found in the outer and innner tissues of the integument with little colouration in the middle integument. Within 24 hours of inoculation the tannin apparently began to diffuse from the cells of the outer integument and into the parenchyma tissue which surrounded it (day 1 of fermentation). By day 3 of fermentation, the epidermal cells were more intensely coloured than the parenchyma tissue, suggesting that tannin from the outer integument had traversed the parenchyma cells and was concentrated in the epidermal cells. The cells of the inner integument remained strongly coloured throughout fermentation. Seeds

extracted on day 5 of fermentation showed a significant depletion of colour in the three cell types: epidermal, parenchyma and the outer integument. Even seeds separated from grape marc at pressing (day 8) retained intense colouration of the inner integument, whereas at this stage the cells of the outer integument showed some loss of integrity of the cell walls, while intact cells were mottled brown in colour indicative of a variation in tannin concentration (Figure 6b.3).

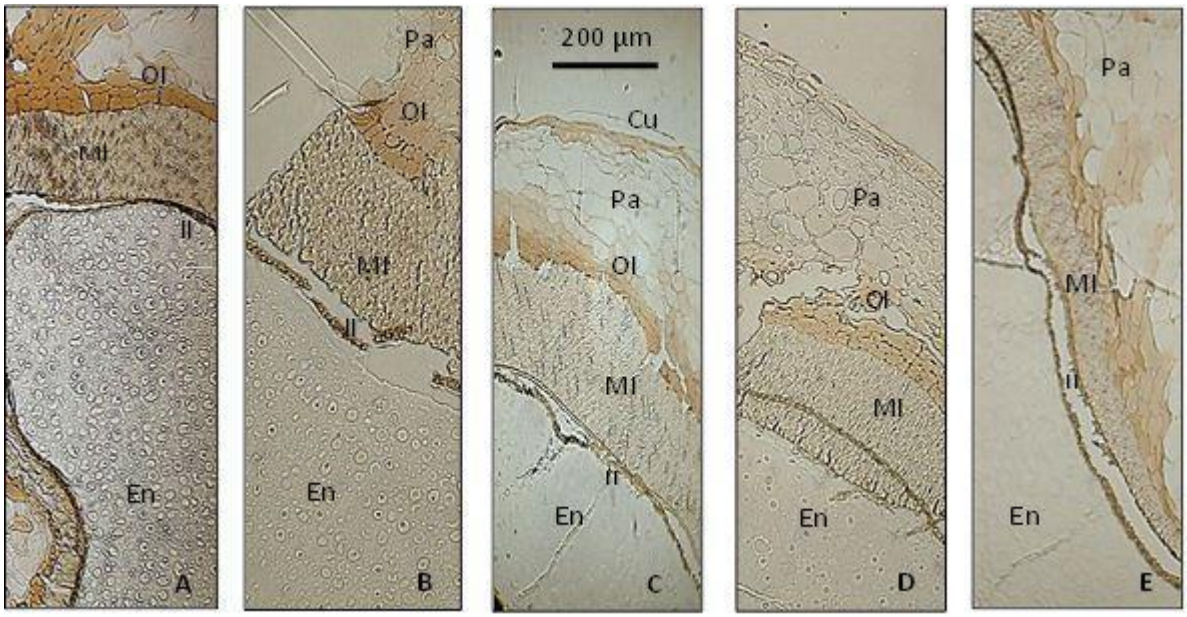

Figure 6b.3. Histochemistry of fermenting Pinot Noir grape seed

Light micrographs of cells of grape seeds sequentially through fermentation pictured in Panels A to E: Panel A = fresh seed at inoculation; B = seeds removed after 1 day (24 hours) of fermentation; C= seeds removed after 3 days; D = seeds removed after 5 days; E seeds separated from grape marc at pressing, following 8 days of fermentation. The flavan-3-ols were stained with vanillin and the concentration of tannin was proportional to the intensity of brown colouration in the cells. The outer integument (OI), and the inner integument (II) were intensely coloured, while parenchyma (Pa) cells and the cuticle (Cu) showed a transition of colour as fermentation progressed. Tissues of the endosperm (En) were not stained by vanillin.

The histological investigation showed that seed tannins started to diffuse from a zone of high conentration in the outer integument on day 1 of fermentation, but it was not until day 5 that tannin loss from the seed itself became apparent.

6b.4.4 Phenolic composition in wine at six months bottle age

Significant changes in the phenolic composition were found in wines from which a proportion of the seeds had been removed during fermentation. The addition of pectolytic enzyme to the musts had less impact on the majority of phenolic parameters than did the seed removal treatment (Table 6b.2).

Table 6b.2 Pinot Noir phenolic parameters for wine at six months bottle age from which released seeds were removed during fermentation; musts were fermented in the absence or presence of Lafase HE pectolytic enzyme.

Phenolic parameter	Anthocyanin (g/L)	Tannin (g/L)	[†]NBP (AU)	[ᾔ]WCD (AU)	Hue (AU)	Hue SO_2 (AU)
<u>No enzyme</u>						
control	0.23 b	0.91 a	1.10 a	6.10 a	0.77 a	1.18 b
remove seeds day 1	0.24 b	0.80 b	0.95 b	5.23 b	0.71 c	1.20 b
remove seeds day 3	0.25 b	0.71 c	0.80 c	4.65 b	0.73 bc	1.31 a
remove seeds day 5	0.28 a	0.73 bc	0.93 b	5.28 b	0.70 bc	1.23 b
<u>Plus enzyme</u>						
control	0.26 b	1.18 a	0.92 b	5.60 a	0.68 c	1.18 b
remove seeds day 1	0.25 b	1.12 a	1.06 b	5.39 a	0.68 c	1.10 c
remove seeds day 3	0.25 b	1.06 b	0.87 b	4.68 b	0.68 c	1.21 b
remove seeds day 5	0.25 b	1.07 b	0.92 b	4.97 b	0.70 bc	1.20 b
[¥]*LSD (trt)*	*NS*	*0.08*	*0.14*	*0.58*	*NS*	*0.07*
P-value	*0.604*	*0.001*	*0.044*	*0.005*	*0.17*	*0.022*
[¥]*LSD (enz)*	*NS*	*0.06*	*NS*	*NS*	*0.02*	*0.05*
P-value	*0.890*	*<0.001*	*0.958*	*0.444*	*<0.001*	*0.023*

Data are treatment means (n=3). [†]Non-bleachable pigment; [ᾔ]Wine Colour Density; [ϰ]Hue (ratio A420:A520 in model wine solution) [ħ]Hue SO_2 (ratio A420:A520 in model wine solution and 0.375% sodium metabisulphite); [¥]Least Significant Difference. Numbers within a column and enzyme treatment with different letters are significantly different at $P \leq 0.05$. NS, Not Significant at $P \leq 0.05$.

Of all the phenolic parameters assessed, differences in tannin concentration were the most profound; the removal of seeds on any of the selected days during fermentation resulted in an average reduction of 22% ($P = 0.001$) in tannin concentration relative to control wines. By contrast, the additon of pectolytic enzyme increased the average tannin concentration by 49% ($P < 0.001$) across all treatments (Table 6b.2).

Although there was no significant difference in anthocyanin concentration between the control and treatment wines, a significant reduction (21%) in the non-bleachable pigment (NBP) content of wines from which seeds were removed on day 3 or 5 of fermentation was observed, relative to the control wines and ferments where the seeds were removed on day 1 of fermentation. There was no reduction in wine colour density (WCD) when seeds were removed on day 1 of fermentation but later seed removal (day 3 or 5) reduced WCD by 20%. The addition of pectolytic enzyme had no significant effect on WCD but lowered the hue of the wine by 7%, which gave the wine a more blue-purple colouration. Hue SO_2 values of the control wines and those where seeds were removed on day 1, were 8% lower than wines where the seeds were removed on day 3 or 5 of fermentation, again indicative of a greater proportion of stable blue-purple wine pigments. The addition of pectolytic enzyme also lowered wine hue SO_2 values, but to a lesser extent (5%) than did seed removal (Table 6b.2).

The response in the spectral profile of treatment wines six months post bottling was assessed across the ultraviolet range from 200 nm to 300 nm (Figure 6b.4). The most significant changes noted in the spectra were the peaks occuring at 220 nm and 280 nm wavelength.

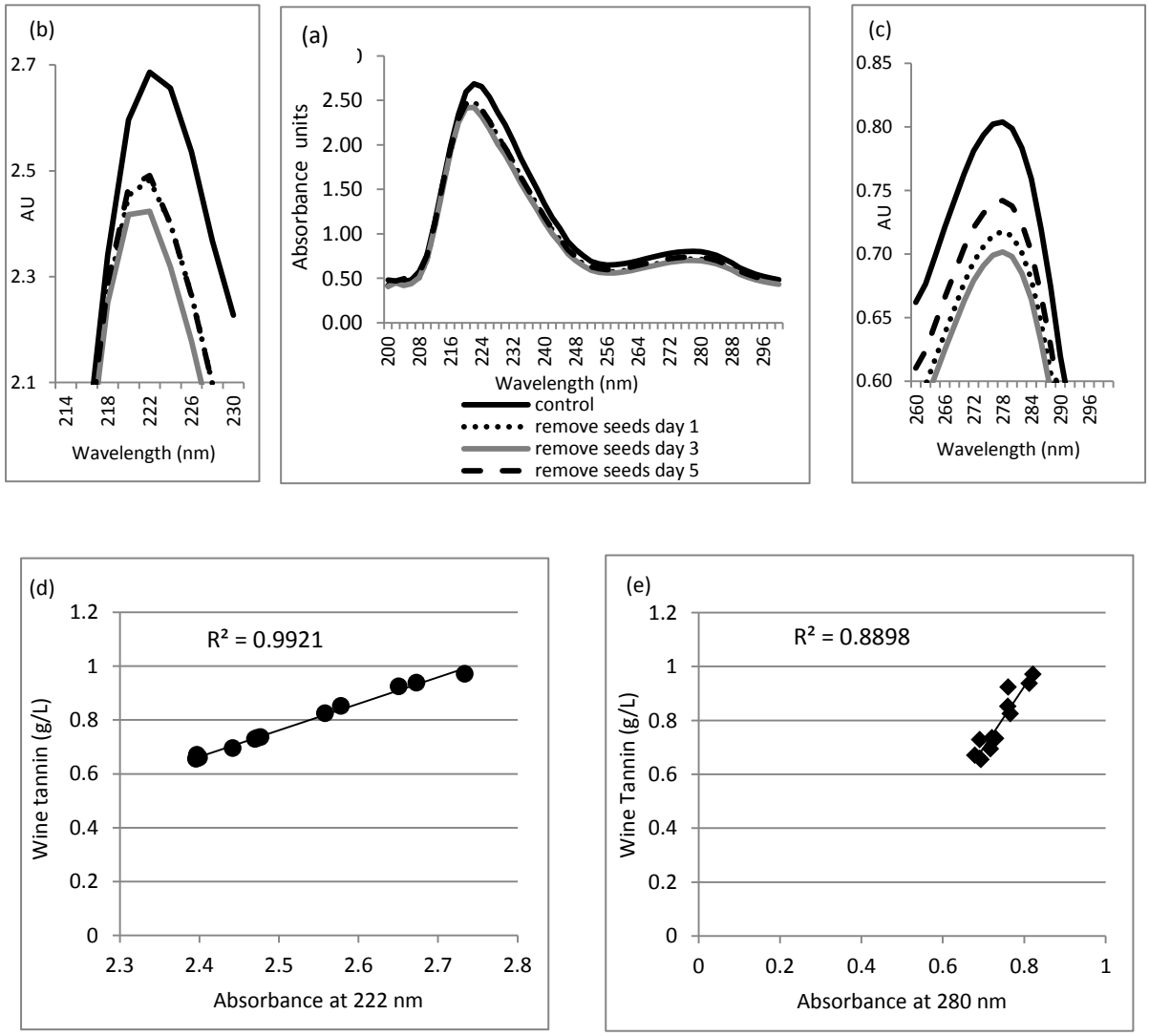

Figure 6b.4. Comparison of ultraviolet absorbance of wines at six months bottle age made with full complement of seeds (control) and wines from which seeds were removed on day 1, 3 or 5 of fermentation.

Wines were diluted 1:51 in 1.0 M HCl to assess wine phenolics using Rapid Tannin Assay (Dambergs et al., 2012b) (a) absorbance at 220 nm (expanded scale); (b) absorbance spectra from 200 to 300 nm wavelength; (c) absorbance at 280 nm (expanded scale); (d) relationship between peak absorbance of Pinot Noir wine at 220 nm wavelength and tannin concentration (*P*=0.003); (e) relationship between peak absorbance of Pinot Noir wine at 280 nm wavelength and tannin concentration (*P*=0.003).

Absorbance at 220 nm decreased by 9% (P=0.030) when seeds were removed from the ferment, and was independent of the day on which seed removal took place. When seeds were removed, absorbance at 280 nm decreased by 12% (P=0.004) and was also independent of the day of seed removal. In summary, the changes in peak height at these wavelengths in wines where seeds were removed were significantly different from the control wines, regardless of the day on which the seeds were removed.

Linear regression analysis at these wavelengths showed significant relationships between the absorbance peaks and tannin concentration of the wine, calculated by spectroscopy and chemometrics (Dambergs et al., 2012b), with absorbance at 220 nm showing the strongest correlation with seed tannin concentration which declined as seeds were removed from the ferment ((d) and (e) in Figure 6b.3).

6b.5 DISCUSSION

The study found that only 15% of the seeds had been released from the pomace cap on day 1 of the ferment, yet the removal of those seeds had the greatest impact on the phenolic composition of the wines. Less seed contact time produced wines that at six months bottle age were considerably less tannic than control wines and had a higher proportion of stable blue-purple pigmentation, which suggested the presence of a particular class of pyranoanthoycanins known as portisins, which have blue-purple colouration (Boulton, 2001, Alcalde-Eon et al., 2006, Cheynier et al., 2006). By contrast, the addition of pectolytic enzyme resulted in much higher concentration of tannin in the wines, yet some increase in the blue-purple colouration was also observed.

Histological images demonstrated the progess of depletion of tannin from the outer integument of the seed coat during the eight-day fermentation period. By the end of the

fermentation, only 35% of the total number of seeds in the must had been released from the pomace, the remainder being retained in the pomace cap or suspended in the wine matrix. Of this 35%, almost half were released within 24 hours of inoculation, and their removal had a significant effect on wine tannin concentration and wine hue at six months bottle age.

The addition of pectolytic enzyme to red wine must is generally practised to increase the rate of grape cell tissue breakdown, specifically to improve the extraction of colour pigments and tannins from grape tissues into the wine matrix, however from which of the grape tissues the tannin is extracted is poorly defined. Whilst it was anticipated that the addition of pectolytic enzyme to the treatments involved in this study might cause a greater number of seeds to be released from the pomace as grape pulp structures weakened, this apparently was not the case. Instead, there was a slight increase in the number of seeds released on the first day of fermentaton, but on day 3 significantly fewer grape seeds were released compared with the parallel treatment with no enzyme. By day 5 of fermentation, the percentage of seeds released from the pomace cap without enzyme was similar to treatments with pectolytic enzyme, suggesting that seed release was not necessarily promoted by the activity of pectolytic enzymes. Hence, it appeared that towards the latter stages of fermentation, the integrity of the pulp tissues had deteriorated to the same extent in both treatments.

A comparison of wine phenolic composition and histochemistry of released seeds provided further insights. Considering that 15% removal of seeds on day 1 of fermentation coincided with a 14% reduction in wine tannin, it appeared from the cellular images that tannin had diffused from the cells of the outer seed integument within 24 hours of inoculation, but had become localized within the parenchyma cells of the seed coat between the integument and

the epidermis. Consequently, the removal of these seeds on day 1 of fermentation represented a net loss of 14% of tannin in six month old wines. The middle integument of the seed is very hard (Cadot et al., 2006), and may have prevented the phenolic compounds of the inner integument from being extracted during winemaking. However, the mobilisation of tannin from the tissue of the outer integument observed in the early stages of fermentation confirms previous speculation that the extraction of tannin from the seed may be unrelated to the alcohol concentration of the fermenting must, and be a consequence of the decline in the integrity of the external tissues of the seed (Busse-Valverde et al., 2010, Zanchi et al., 2007).

Linear regression analysis showed that ultraviolet absorbances at 220 and 280 nm were indicators of phenolic compounds from the seed. While evidence of absorbance by tannins at 280 nm has been well documented (Garcia-Jares and Medina, 1995, Skogerson et al., 2007, Dambergs et al., 2012b), the additional response peak 220 nm has more frequently been associated with the proanthocyanidin subunits and the grape derived caffeic acid and gallic acids. Both galloylated proanthocyanidins and gallic acid are present at higher concentration in grape seeds than in grape skins, while neither epi-gallocatechin nor caffeic acid are present in grape seeds (Robbins, 2003, Yilmaz and Toledo, 2004, Cortell et al., 2005, del Rio and Kennedy, 2006).

In their work with the addition of seed oenotannins to red grape ferments, Neves et al. (2010) reported an increase in the concentration of gallic acid in the wine to which oenotannins were added, yet no gallic acid was detected in the oenotannin itself. This suggests that gallic acid may have been formed as a by-product of degradation or polymerisation of seed proanthocyanidins as suggested in Chapter 6a. As the ratio of seed tannin to skin tannin is approximately 3:1 in Pinot Noir grapes (Cortell et al., 2005, Cortell et

al., 2007, Cortell and Kennedy, 2006, Kemp et al., 2011) it seems likely that the proanthocyanidin subunits catechin, epicatechin and epicatechin gallate and gallic acid may be the major contributors to the peak absorbance at 220 nm observed in this study and would explain the strong correlation with tannin concentration. It is noteworthy that, in addition to condensed tannins, gallic acid was recognised in early medical-botanical literature as a component of plant galls imparting a bitter character to the plant tissue (Paris, 1825). Consequently, it is possible that gallic acid from grape seed may partly account for the bitter flavour in wine that is generally associated with seed tannins (McRae and Kennedy, 2011, Noble, 1998, Vidal et al., 2003a).

6b.6 CONCLUSION

This investigation described the response of young Pinot Noir wines to the removal of grape seed during fermentation and demonstrated that seed removal has a significant impact on wine phenolic composition, however the timing of seed removal made little difference provided it took place within the first 5 days of fermentation. Focussing on the potential for commercial adoption, in this experiment only those seeds that had fallen to the base of the fermentation vessel were removed. Histological observations confirmed that phenolic release is due to decline of seed coat integrity rather than alcohol concentration, and supported the changes observed in the phenolic composition of wines coincident with sequential seed removal. The recognition of peak absorbance at two distinct wavelengths in the ultraviolet spectrum may provide an opportunity for simple differentiation of seed and skin proanthocyanidin derivatives on the basis of the level of their galloylation. As the method used for preparing the wine for this assay was both rapid and inexpensive, there may be potential to develop a commercial application of this technique to assist in crucial

decision making processes during vinification. Improvements in the phenolic composition, and by implication the colour and flavour characteristics of Pinot Noir during the fermentation phase of vinification, by optimising the extraction of skin derived tannins over seed derived tannins is an area worthy of further investigation.

6b.7 SUBSEQUENT RESEARCH FOCUS

The impact of seed tannin on Pinot Noir wines provided valuable insights into the differences in wine phenolic composition afforded by one of the major grape berry components. As an excess of seed tannin was shown to be detrimental to wine quality and the removal of seeds to have some benefits, a radical change in winemaking procedure may be beneficial. As the rate of seed release from grape pomace was found to be relatively slow, an alternative procedure considered was one in which the the influence of skin tannin was exacerbated, so that it could more readily compete with seed tannin for binding sites with anthocyanin. An increase in the proportion of skin tannins in Pinot Noir wine may also reduce the incidence of bitter flavours. This hypothesis was investigated in experiments described in Chapter 7.

7

7A ACCENTUATED CUT EDGES (ACE): INFLUENCE OF REDUCED SKIN PARTICLE SIZE ON PHENOLIC EXTRACTION FROM PINOT NOIR GRAPES

Preliminary work described in this chapter, was awarded best student poster at 8th International Cool Climate Symposium, held in Hobart, Tasmania, January 2013

Title: Is Pinot Quality Skin Deep?

Authors: Angela M Sparrow, Richard E Smart and Robert G Dambergs

7a.1 ABSTRACT

Modifying the phenolic composition of Pinot Noir wines can lead to increased ageing potential, overcoming a common defect. This study compares two maceration techniques: in one, pectolytic enzymes were added to the fermenting must to promote the breakdown of cellular integrity enhancing release of phenolic components from the grape tissue and in the second, the skin particle size was reduced by cutting the skins into small fragments, thereby increasing the perimeter of grape skin edges per unit surface area (Accentuated Cut Edges; ACE). This process also facilitated greater egress of colour and tannin phenolics from the skin. The inclusion of pectolytic enzyme in the fermenting must was found to increase the concentration of tannin in the wine by 17% and blue purple hues of the wine by 6%, with no effect on either wine colour density or stable pigment components. By contrast, reducing the particle size of the skins to approximately 4% of the original size (ACE maceration) resulted in a 3-fold increase in the tannin content. When applied one day after inoculation, ACE maceration also resulted in a 94% increase in non-bleachable pigment (stable pigments), and a 49% increase in wine colour density. ACE treatment was significantly less effective when applied three or five days after inoculation. The blue-purple colouration of the wines increased by 18% with ACE treatment and was independent of the day the treatment was applied. The results demonstrate that ACE maceration had a substantial and positive impact on the phenolic composition of Pinot Noir wines, much more

so than the addition of pectolytic enzyme. This novel technique could be incorporated into commercial wine making practices with Pinot Noir and represents a viable alternative to the existing maceration techniques commercially practiced.

7a.2 INTRODUCTION

Fermentation of red wine grapes releases chemical compounds into the wine matrix firstly by diffusion and later by dissociation of cellular membranes. If the cells are physically ruptured at crushing, cellular components also leak from the edges of the torn tissues. Wine tannins (condensed tannins or proanthocyanidins) are the most abundant class of phenolic substances found in the skin and seeds of the wine grape *Vitis vinifera* (Singleton, 1992). Their structure is a result of the polymerisation of flavan-3-ol subunits and varies depending on whether the tannins are derived from grape skins, seeds or stems (Vivas et al., 2004, Herderich and Smith, 2005, Prieur et al., 1994). In addition to the tactile sensation of astringency attributed to wine tannins (Gawel, 1998, Sun et al., 2001, Vidal et al., 2003a, McRae and Kennedy, 2011), there is a complementary interest in the ability of wine tannins to form covalent adducts with anthocyanins, thereby stabilising the colour of the anthocyanins (Remy et al., 2000, Boulton, 2001, Sacchi et al., 2005). These anthocyanin-tannin adducts, (or 'pigmented tannins') have an impact on the wine's ability to age favourably, as they become involved in condensation and oxidation reactions (Cheynier 2006). Recent research has indicated that the relative proportion of seed tannin to skin tannin in red wine can influence wine quality. The formation of anthocyanin-skin tannin adducts has been associated with a higher wine sensory score determined by a sensory panel, than the formation of anthocyanin-seed tannin adducts in Shiraz wines (Ristic et al., 2010).

The type of maceration technique used in commercial practice determines the nature of contact between the pomace and grape juice or fermenting wine. Maceration techniques aim to optimise extraction of anthocyanins and tannins from red wine grapes and include: cold soaking, SO_2 addition, thermo-vinification, grape or must freezing, pectolytic enzyme addition, carbonic maceration and extended maceration (Spranger et al., 1998, Yokotsuka et al., 2000, Sacchi et al., 2005, Villaño et al., 2006). As described in Chapter 3, the maceration technique chosen is particularly important when making wine from *Vitis vinifera* cv. Pinot Noir which is known for its poor colour extraction, low pigment stability and low ratio of skin to seed flavanols (Sacchi et al., 2005, Neves et al., 2010). For example, in Pinot Noir grapes the ratio of skin to seed flavanols is 1 to 63; in Cabernet Sauvignon it is 1 to 11 and in Shiraz, the ratio is 1 to 7.5 (Mattivi et al., 2009). While anthocyanins are extracted in the early stages of maceration and reach their maximum amount before the start of alcoholic fermentation, tannins are generally extracted later in the fermentation and continue to increase in concentration until the end of fermentation (Spranger et al., 1998, Ribéreau-Gayon, 1982). Recent studies have demonstrated that it is not necessarily the increase in alcohol concentration that causes tannins to be extracted later in the fermentation as once thought, rather, the breakdown of the polysaccharide structure of pulp tissues and the loss of seed coat integrity are thought to facilitate tannin extraction (Watson et al., 2000, Sacchi et al., 2005, Gambuti et al., 2009).

In one of the techniques used in Pinot Noir winemaking, a proportion of whole grape bunches is included in the ferment to effect carbonic maceration (Flanzy, 1935). This assists in the production of a heavy bodied wine due to the extraction of tannins from the stalk. Other maceration techniques that do include the removal of bunch stems may eliminate the berry crushing step with the intention of maximising fruit flavour and aroma in the wine to

create a style intended to be consumed young. Nonetheless, minimal berry crushing is also likely to compromise the extraction of colour from the grape skins (Somers, 1971) and the extraction of tannin from both skin and seed of the grapes (Pinelo et al., 2006).

Pectolytic enzymes have also been used to enhance phenolic extraction during the maceration phase of winemaking. These act by increasing the rate of grape cell tissue breakdown, releasing colour pigments and tannins from grape tissues (Pardo et al., 1999, Bautista-Ortín et al., 2005, Ducasse et al., 2010b, Sacchi et al., 2005). Meyer et al. (1998) noted that reducing the particle size in the pomace improved pectolyitc enzyme activity.

This study describes an innovative wine making technique in which the grape skin particle size is reduced by mechanically cutting the grape pomace part way into fermentation. The ratio of skin edge to skin area increases in response to the decrease in skin fragment size. The cutting method used in the experiments described here caused minimal damage to the grape seeds. Experiments described here evaluated the timing of tannin and anthocyanin release from grape skin tissues into the wine matrix of ACE wines, as earlier experiments (Chapter 4) suggested that this might optimise the formation of anthocyanin-skin tannin adducts prior to the extraction of seed tannins.

A sequence of experiments to evaluate ACE maceration are described in this study: A. Reduce grape skin particle size by homogenisation; B. Reduce skin particle size with minimal damage to seeds and; C. Reduce skin particle size at different stages of fermentation with no seed damage. In conjunction with experiment C, the impact on phenolic extraction in the absence of seeds and in the presence of pectolytic enzyme was also investigated.

7a.3 MATERIALS AND METHODS

To evaluate the detailed treatment effects of the experiments that follow, Submerged Cap micro-vinification techniques were used that employed either 1.5 L or 250 mL fermentation vessels. Micro-vinification vessels of this size when used in previous experiments, were found to provide reproducible fermentation conditions and wine composition results comparable to the more widely adopted 20 L ferments (Becker, 1968, Becker and Kerridge, 1972, Antcliff and Kerridge, 1975, Dambergs and Sparrow, 2011, Smart et al., 2012, Sparrow et al., 2012b, Sparrow et al., 2012c, Sparrow et al., 2013a, Sparrow et al., 2013b, Sparrow et al., 2013c, Sparrow et al., 2014).

7a.3.1 Grape sampling and analysis

Grapes of *Vitis vinifera* cv. Pinot Noir from a single vineyard located in northern Tasmania, Australia were used for each of the experiments described. Experiments A and B were conducted in April 2011 using Clone G5V12, and experiment C was conducted in April 2012 using Clone 115. Each experiment was conducted and analysed autonomously. Four replicates of each treatment were conducted for all three experiments. Grape bunches were randomly assigned to each replicate, prior to de-stemming and crushing the fruit. A sub-sample of 100 berries from each replicate was selected at random, hand-crushed and the juice drained to determine total soluble solids (°Brix), measured using a hand-held refractometer; pH, using a Metrohm pH meter/autotitrator; and titratable acidity by titration with 0.333 M NaOH to an end point of pH 8.2, reported as g/L tartaric acid. A further 200 g sub-sample of berries from each replicate was frozen at 20 ^{o}C for later analysis of grape colour (Iland et al., 2004) and tannin (Dambergs et al., 2012b).

7a.3.2 Treatments for winemaking

Experiment A. Pomace homogenisation and seed removal:

Crushed berry treatments were compared with crushed berries followed by the removal of seeds that been released from fermenting grape pomace. These in turn were compared with crushed berries in which the fermenting pomace was homogenised to a paste using a Waring blender (CAC33). The homogenisation procedure was also applied in the presence or in the absence of grape seeds. Homogenisation was intended to maximise the extraction of tannins from the grapes by creating very small berry fragments relative to those of crushed berries. Seed removal was intended to assess the impact of reduced skin particle size in the absence of seeds. Treatments for this experiment were: (1) crush berries (Control); (2) crush berries, separate and remove free seeds; (3) homogenise skins and return separated seeds; (4) remove and discard separated seeds then homogenise skins; (5) homogenise seeds and skins. For each treatment replicate, 1 kg of Pinot Noir berries were crushed by hand and placed in a 1.5 L Bodum® coffee plunger. Sulphur dioxide was added in the form of potassium metabisulphite solution at 50 mg/L and the must refrigerated overnight at 4^{o}C. In order to optimise seed release from the pomace, a method was developed to facilitate pulp tissue breakdown by increasing the concentration of pectolytic enzyme and yeast at the beginning of fermentation: chilled grape musts were equilibrated to 25^{o}C and lightly pressed to remove 400 mL juice which was returned to 4^{o}C. The grape must was then fermented using twice the usual rate of yeast and pectolytic enzyme (600 mg/L yeast RC212 and 80 mg/L Laffort Lafase HE Enzyme) which were mixed thoroughly into the juice depleted ferments and incubated at 27± 1^{o}C. Approximately 200 mL of liquid remained in these musts. On day 3 of fermentation a slotted spoon was used to remove the floating pomace cap from the ferment and place it in a sieve to drain.

According to the treatments outlined above, the seeds that had fallen to the bottom of the fermentation vessel were either discarded or recombined with the drained pomace and the 200 mL of fermenting juice. The mixture was blended in a Waring Laboratory Blender 7009S/7009G, using three pulses each of 25 seconds, which reduced the grape solids to a homogeneous paste. Subsequently, the 400 ml of juice that had been poured off was brought to 25^{o}C and recombined with the pomace paste, and fermentation continued in the 1.5 L Bodum® French Press coffee plunger at 27± 1^{o}C.

Experiment B. Crushed versus cut berries:

This experiment investigated the effect of eliminating the extensive handling of the fermenting must by reducing the grape particle size, prior to inoculation without the use of pectolytic enzyme. Treatments for this experiment were: (1) crushed berries (Control) and (2) cut berries. For the Control treatment, 200 g of berries were hand crushed; for the cut berries treatment, crushed grapes were placed in a Waring Laboratory Blender 7009S/7009G and subjected to three, one second pulses which significantly reduced the particle size of the skins, yet inflicted minimal damage on the seeds. Fermentation was conducted in 250 mL polycarbonate jars. Samples of marc, post-fermentation, showed that the maximum proportion of seeds showing damage in the 'cut berries' treatment was 8% of the total seed number (244) from 200 g of grapes

Experiment C. Timing of pomace cutting and influence of pectolytic enzyme:

As tannins tend to be extracted more readily as the integrity of the grape tissues weaken, the third experiment was conducted on alternate days of a six-day fermentation period using ACE maceration in a 250 mL fermentation vessel. The application of ACE procedure reduced the skin particle size of the floating pomace cap post-inoculation and caused no damage to the seeds. The ACE procedure was implemented by removal of the spacer and

gauze mat from the fermenting wine that were used to effect Submerged Cap micro-vinification, allowing the pomace cap to re-float. After 1 hour, the pomace cap was removed from the fermenting wine using a gauze sieve (1 mm x 1 mm) and transferred to a separate vessel. The fermenting juice that remained was passed through a second gauze sieve to collect the seeds; the liquid was then recombined with the pomace cap and 10 pulses, each of one second duration, were applied to the mixture with a Russell Hobbs Stick-Mixer® (Salton RHSM650). The rotating blades of this apparatus were shielded by a stainless steel hood 60 mm in diameter, with four 20 mm x 5 mm gaps spaced evenly around the lower edge of the circumference. As the blades rotated, the seeds that were suspended in the pomace cap were displaced by the cutting blades through the gaps in the hood. After ACE maceration, the fermenting must was then returned to the fermentation vessel and the fermentation continued. In treatments where ACE maceration was applied in combination with the removal of seeds, those seeds collected in the second gauze sieve were discarded. In this experiment all seeds remained intact while the skin particle size was significantly reduced. Treatments for this experiment were: (1) crushed berries (Control); (2) ACE day 1; (3) ACE day 3; (4) ACE day 5; (5) remove seeds and ACE day 1; (3) remove seeds and ACE day 3 (4) remove seeds and ACE day 5. Replicates of the crushed berry Control treatment were treated in the same way but the cap was not subjected to ACE maceration nor the seeds removed.

To compare the effect of pectolytic enzymes on berry tissue breakdown, each of these treatments was repeated with the inclusion of 40 mg/L Laffort Lafase HE pectolytic enzyme in the must. The enzyme was added two hours prior to yeast inoculation.

7a.3.3 Calculation of ACE effect:

At the conclusion of fermentation, marc samples were collected from each treatment in Experiment C, and three sub-samples of 1 g fresh mass were assessed. The sub-samples were floated in water in a glass petri dish (100 mm diameter) to determine the perimeter and surface area of the cut skins. When whole berries are crushed, as for the control treatment, the skins form a deflated sphere with a double V-shaped edge (Figure 7a.1). The average diameter of berries was 13.0 mm ± 0.1 mm (n=18) and the measured length of broken skin edges due to crushing was 28.0 mm ± 0.8 mm. Assuming that the berry was spherical, the mean berry surface area was calculated at 531 mm^2 and when crushed, the ratio of skin edge to surface area was 28.0 mm/531 mm^2 = 0.053 mm^{-1}.

For the calculation, skin fragments (n=346) measured after ACE maceration were assumed to be rectangular in shape and cut skin edges averaged 5.9 ± 0.4 mm in length and 3.8 ± 0.3 mm in width, giving a perimeter of 19.4 mm, a surface area of 22.4 mm^2 and a ratio of skin edge to surface area of 19.4 mm/22.4 mm^2 = 0.86 mm^{-1} which was 16-fold greater than the same ratio for the crushed berries.

Based on the mean surface area per cut skin fragment, there were 23.7 skin fragments per berry, giving a total length of cut skin edges of 460 mm, so that the length of cut edges in ACE treated berries had increased 16.4-fold relative to the crushed berries.

Our theoretical studies have shown that the ratio of skin edge to surface area increases asymptotically with fragment number, so that excessive fragmentation is unnecessary.

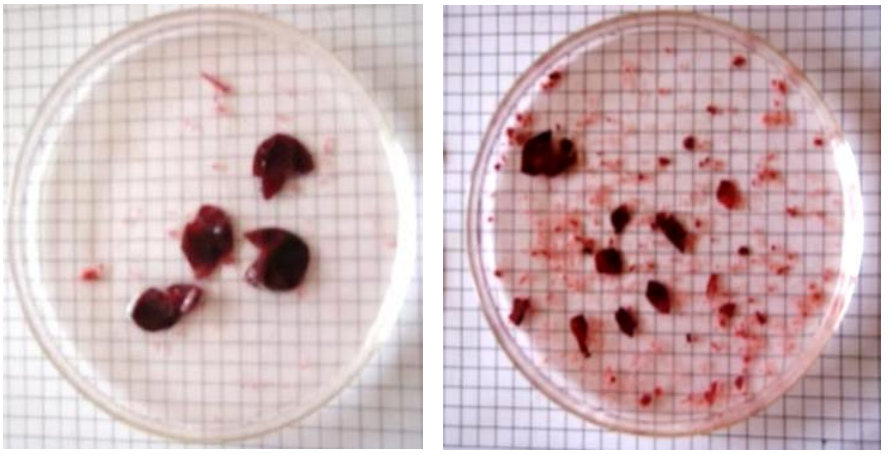

Figure 7a.1. Fragment size of crushed berry (Control) and ACE macerated grape skins (marked squares: 5 x 5 mm).

7a.3.4 Vinification protocol:

After preparing the musts for fermentation, 50 mg/L SO_2 was added to each fermentation vessel as potassium metabisulphite solution. The must preparations equilibrated to ambient temperature were inoculated with 300 mg/L RC212 yeast solution and fermented under Controlled temperature conditions at 27°C (±1°C). Fermentation vessels were covered with a loose fitting lid and subject to submerged cap fermentation (Dambergs and Sparrow, 2011, Smart et al., 2012). On day three of the fermentation, 300 mg/L of diammonium phosphate was added to each fermentation vessel. At day eight, each ferment was complete and confirmed to contain less than 2 g/L of residual sugar using Clinitest™ reagent tablets (Bayer Australia Ltd.). The wine from each ferment size (200 g and 1 kg ferments) was pressed using a plunger with a gauze sieve (element size 1 mm x 1 mm) and enough pressure was applied to recover 60% (v/w) of the must weight (120 mL and

0.6 L of wine respectively). Wine was stored in amber glass screw topped bottles at 4°C for 14 days to settle lees, at which time it was racked under CO_2 cover and a further 80 mg/L SO_2 added. After storage for a further 30 days at 12°C wine was racked a second time under CO_2 cover to amber glass bottles and sealed with screw caps. Samples were taken for phenolic analysis at bottling (50 days after inoculation) and at six months bottle age (230 days after inoculation).

7a.3.5 Quantifying the effect of tannin supplements on wine phenolics

Samples were clarified by centrifugation at 500 rpm for five minutes using an Eppendorf 5417C centrifuge with an Eppendorf F45-30-11 rotor (Crown Scientific, Australia). The phenolic composition of all samples was determined using the modification of the Somers assay described by Mercurio et al. (2007) and rapid tannin analysis (Dambergs et al., 2012b). Wine samples were diluted in each of three solutions (Assay A: 0.1% (v/v) acetaldehyde in model wine buffer; Assay B: 0.375% sodium metabisulfite in model wine buffer and Assay C: 1 M hydrochloric acid). For all assays, the samples were mixed by inverting screw capped tubes three times and incubated in the dark at room temperature. Wine samples analysed using Assays A and B were incubated for 1 h at room temperature. Assay C samples were incubated at room temperature in the dark for 3 h. Absorbances of all samples were read at 2 nm intervals from 200 to 600 nm using an Ultraviolet-Visible (UV-Vis) Spectrophotometer (Model Genesys™ 10S Thermo Fisher Scientific Inc., Madison, WI, USA) that had been calibrated for rapid measurement of methyl cellulose precipitable tannins by UV-VIS spectrophotometry and chemometrics (Dambergs et al., 2012b). Wine phenolic parameters calculated from these assays were free anthocyanin, total tannin, non-bleachable pigment, colour density, hue and hue SO_2. Free anthocyanin refers to anthocyanins in solution that

have not become chemically bound to other molecules and appear red at low pH. Non-bleachable pigments are those pigments that are resistant to bleaching in the presence of sulphur dioxide and consist of either polymerised anthocyanin molecules (pyranoanthocyanins) (Boulton, 2001, Cheynier et al., 2006) or molecules in which one or more anthocyanin molecules have become bound to proanthocyanidins (condensed tannins) to form 'pigmented tannins' (Harbertson et al., 2003). Wine hue is calculated from the ratio of absorbance of the sample at 420 nm to its absorbance at 520 nm in model wine solution containing 0.1% (v/v) acetaldehyde and generally increases with age as free anthocyanins become bound within pigmented tannins, to form red-brown pigments (Somers, 1971). Hue SO_2 is calculated from the ratio of the absorbance of the sample at 420 nm to its absorbance at 520 nm in model wine solution containing 0.375% sodium metabisulphite and is a measure of the hue of the wine pigment resistant to sulphur dioxide bleaching; a low hue SO_2 is indicative of stable colour shifted towards the blue-purple spectrum, as opposed to red-orange, and may be indicative of a larger proportion of flavanyl-vinyl-pyranoanthocyanins (portisins) which are blue-purple in colour (Alcalde-Eon et al., 2006, Cheynier et al., 2006).

7a.3.6 Statistical analysis

Mean, standard deviation and co-efficient of variation were calculated for each grape compositional parameter. Statistical analyses on wine phenolics were conducted using GenStat 64-bit Release 16.1 Copyright 2013, VSN International Ltd. For experiment A, data for individual phenolic parameters of the wine samples were analysed using Repeated Measures ANOVA, with treatment as the main plot and time as the sub-plot. For experiment B, data for individual phenolic parameters of the wine samples were analysed

using one-way ANOVA. For experiment C wine samples were analysed using Repeated Measures ANOVA, with treatment as the main plot and inclusion of pectolytic enzymes as sub-plots. For each of the phenolic parameters assessed, ANOVA was followed by post-hoc analysis using Fisher's Protected Least Significant Difference (LSD) test.

7a.4 RESULTS

7a.4.1 Grape composition:

The berry composition and phenolic content of grapes used in this study are listed in Table 7a.1. The coefficient of variation (CV) for the majority of grape composition parameters was less than 5%. Total tannin content of the fruit used in 2011 *Vitis vinifera* cv. Pinot Noir clone G5 V15 had the highest CV (15%; n=4) which is most likely to have been a function of the variation in berry weight (CV 13%).

Table 0.4 Composition of *Vitis vinifera* cv. Pinot Noir grapes. Data are mean, standard deviation (STDEV), and co-efficient of variation (CV%) (n = 4).

Berry parameter	Clone G5V15 2011			Clone 115 2012		
	Mean	STDEV	CV%	Mean	STDEV	CV%
Berry Weight (g FW‡)	1.0	0.12	12	1.14	0.00	0.0
Sugar (^{O}Brix)	21.0	0.28	1.3	22.4	0.72	3.2
pH	3.40	0.12	3.5	3.14	0.03	0.8
Titratable acidity (g/L)	10.1	0.26	2.5	9.00	0.21	2.4
Anthocyanin (mg/g FW‡)	0.38	0.04	10	0.63	0.07	11
Total tannin (mg/g FW‡)	4.68	0.68	15	7.76	0.40	5.2
Total phenolics (AU/g)	0.83	0.10	13	1.30	0.06	4.5

‡FW, fresh weight

The anthocyanin and total tannin concentration of the berries of *Vitis vinifera* cv. Pinot Noir clone G5V15 used in experiments A and B conducted in 2011 were on average 40% lower than in the berries of Pinot Noir clone 115 used for experiment C conducted in 2012.

7a.4.2 Experiment A: Pomace homogenisation and seed removal

Reducing the size of berry fragments by homogenisation of either the pomace (skins and seeds), or the skins alone, resulted in significant differences in wine phenolic parameters. Changes in phenolic parameters followed a similar pattern for each treatment from bottling to six months of bottle ageing, consequently only the changes attributed to treatment effects at six months bottle age have been reported (Table 7a.2).

Table 7a.2 Treatment means (n=4) of Pinot Noir wine phenolic parameters for five maceration treatments at 6 months bottle age. Experiment A.

Phenolic parameter	Anthocyanin (g/L)	Tannin (g/L)	†NBP (AU)	⁋WCD (AU)	Hue	ħHue SO_2
crush berries (Control)	0.09 a	0.45 c	0.48 c	2.60 c	0.79	1.38 ab
crush berries, remove seeds	0.08 a	0.27 c	0.38 c	2.09 d	0.80	1.49 a
homogenise skins	0.08 a	1.93 b	0.68 ab	3.30 ab	0.79	1.22 b
homogenise skins, remove seeds	0.07 ab	1.99 b	0.62 b	3.03 b	0.82	1.31 ab
homogenise skins and seeds	0.06 b	2.66 a	0.73 a	3.46 a	0.78	1.19 b
¥LSD (0.05)	0.02	0.34	0.11	0.38	NS	0.18
P-value	0.009	<0.001	<0.001	<0.001	0.646	0.021

†Non-bleachable pigment; ⁋Wine Colour Density; ħHue resistant to sulphur dioxide bleaching; ¥Least Significant Difference. Results within a column with different letters are significantly different at P ≤ 0.05. NS, Not significant at *P* ≤ 0.05.

Anthocyanin: The treatments that involved a reduction in both skin and seed particle size, resulted in anthocyanin concentrations that were 33% (*P*=0.035) lower than the crushed berry control wine concentration of 0.09 g/L.

Tannin: The tannin concentration of the crushed berry Control wines at 6 months bottle age was 0.45 g/L. Reducing the particle size of the skins by homogenisation resulted in a 4.4-fold (*P*<0.001) increase in total tannin both in the presence and absence of seeds, while homogenisation of skins and seeds together resulted in a 6-fold increase in total tannin (Table 7a.2). Removing the seeds without reducing the particle size of the skin had no significant impact on the tannin concentration of the wine relative to the crushed berry Control.

Non-bleachable pigment (NBP): Treatments in which the particle size of skins was reduced resulted in wines with an average increase in NBP of 41% (P<0.001) relative to the crushed berry Control wine. Removal of seeds from the must had no significant impact on NBP concentration (Table 7a.2).

Wine Colour Density (WCD): The reduction in skin particle size increased WCD by an average of 27% (P<0.001). When the skins were left intact and the seeds removed, WCD was 24% lower (P<0.001) than for the crushed berry Control (Table 7a.2).

Hue and hue SO_2: All wines showed an average increase in hue of 5% (P=0.002) between bottling and 6 months bottle age, while over the same time period hue SO_2 decreased by 22% (P<0.001) (data not shown). Treatments in which particle size of skins and seeds, or skins alone were reduced, were not significantly different from the control in wine hue (P<0.001). By contrast, hue SO_2 values were 12% lower than the control when skin particle size was reduced, and 14% lower than in the treatments where both skins and seed particle size was reduced (Table 7a.2) conferring a greater proportion of stable blue-purple pigments on the wine.

Experiment A results showed that reducing the skin particle size to a paste by homogenisation increased the tannin, stable pigment, colour density and blue-purple hue of the wine relative to the crushed berry control wines. When both tissue types were homogenised together wine tannin concentration increased by a further 36%. The only parameter that was significantly affected by the removal of seeds was the wine colour density, which was significantly less than that of the crushed berry Control wines.

7a.4.3 Experiment B: Crushed versus cut berries

Wines from this experiment were assessed at the time the wine was bottled. Reducing the particle size of the grape skins with minimal seed damage prior to inoculation caused significant differences in the concentration of tannin, non-bleachable pigment, colour density and hue SO_2 of the wine (Table 7a.3). Tannin concentration increased 6.5-fold (P<0.001) relative to the crushed berry Control wines, non-bleachable pigment increased by 71% (P<0.001) and colour density by 62% (P<0.001). By contrast, hue SO_2 decreased by 11% (P = 0.04). Anthocyanin concentrations and hue values of wines from each treatment were not significantly different at bottling. Experiment B showed that reducing the particle size of grape skins resulted in wines at bottling that were more tannic, had greater colour density and more pronounced blue-purple colouration than the crushed berry control wines.

Table 7a3. Treatment means (n=4) of Pinot Noir wine phenolic parameters for two maceration treatments at bottling (50 days) post inoculation.

Phenolic parameter	Anthocyanin (g/L)	Tannin (g/L)	†NBP (AU)	⁋WCD (AU)	Hue (AU)	♄Hue SO_2 (AU)
crushed berries (Control)	0.19	0.30 b	0.31 b	2.53 b	0.71	1.69 a
cut berries	0.20	1.95 a	0.53 a	4.10 a	0.74	1.53 b
¥LSD (0.05)	NS	0.18	0.05	1.50	NS	0.16
P-value	0.538	<0.001	<0.001	<0.001	0.209	0.04

†Non-bleachable pigment; ⁋Wine Colour Density; ♄Hue resistant to sulphur dioxide bleaching; ¥Least Significant Difference; results within a column with different letters are significantly different at P ≤ 0.05. NS, Not significant at P ≤ 0.05.

7a.4.4 Experiment C: Timing of ACE application and influence of pectolytic enzyme

ACE maceration reduced the skin particle size to 4% of the original surface area consequently increasing the perimeter of skin edges by 16-fold, yet caused no observable damage to the seeds.

A number of significant interactions between treatment, enzyme addition and/or bottle age were observed in this study, however only those treatment effects with the potential for practical application in commercial wineries are described in detail. As changes in phenolic parameters for each treatment followed a similar pattern from bottling to 6 months bottle age (230 days post inoculation) and the addition of pectolytic enzyme had minimal effect on these parameters, only the changes attributed to treatment effects at 6 months bottle age are listed in Table 7a.4.

Anthocyanin: The anthocyanin concentration of all treatments decreased by 40% ($P<0.001$) from bottling to six months bottle age, and was independent of the enzyme status (data not shown). ACE maceration resulted in an average decrease in anthocyanin concentration of 23% ($P\leq0.001$) relative to the crushed berry Control wines (Table 7a.4).

Tannin: At six months bottle age, the average increase in wine tannin concentration was 3-fold higher (3.91 g/L) following ACE maceration compared with the crushed berry Control wine (1.05 g/L) regardless of the enzyme status of the wine. When considered separately the the inclusion of pectolytic enzyme in the must increased the tannin concentration of both Control and ACE treated wines by 17 % ($P<0.001$) and was independent of the day ACE maceration was applied (see Appendix 7a.2).

Non-bleachable pigment (NBP): The NBP content of each wine increased by an average of 72% ($P<0.001$) from bottling to six months bottle age. However, when compared to the crushed berry Control wines, ACE maceration applied on the first day of fermentation

resulted in an increase of 94% (*P*<0.001) in NBP at six months bottle age , while either the later application of ACE or seed removal caused an average increase of only 35% (*P*<0.001) in NBP (Table 7a.4). The inclusion of pectolytic enzyme in the must made no significant difference to the NBP concentration (data not shown). The experiment showed that ACE maceration applied on day 1 of fermentation was the most effective treatment for increasing the NBP concentration of the wine (Table 7a.4).

Table 7a.4. Treatment means (n=14) of Pinot Noir wine phenolic parameters at 6 months bottle age, for Control versus ACE maceration fermentation. Data are the average of wines fermented in the presence or absence of pectolytic enzymes.

Phenolic parameter	Anthocyanin (g/L)	Tannin (g/L)	[†]NBP (AU)	[¥]WCD (AU)	Hue (AU)	[ħ]Hue SO_2 (AU)
crushed berry Control	0.25 a	1.05 c	1.01 c	5.58 c	0.73	1.20 a
ACE day 1	0.18 c	3.38 a	1.96 a	8.31 a	0.74	1.02 b
ACE day 3	0.19 bc	3.21 a	1.26 bc	6.96 b	0.74	1.15 ab
ACE day 5	0.19 bc	3.07 a	1.36 bc	6.91 b	0.71	1.09 b
ACE, remove seeds day 1	0.21 b	3.29 a	1.47 b	7.37 ab	0.75	1.10 ab
ACE, remove seeds day 3	0.19 bc	3.13 ab	1.44 b	7.40 ab	0.72	1.11 ab
ACE, remove seeds day 5	0.19 bc	2.89 b	1.30 bc	6.83 b	0.71	1.07 b
[¥]LSD (0.05)	0.02	0.37	0.39	1.00	NS	NS
P-value	<0.001	<0.001	<0.001	<0.001	0.61	0.07

[†]Non-bleachable pigment; [¥]Wine Colour Density; [ħ]Hue resistant to sulphur dioxide bleaching; [¥]Least Significant Difference. Results within a column with different letters are significantly different at P ≤ 0.05. NS, Not significant at P ≤ 0.05.

Wine colour density (WCD): All treatments showed an average increase in WCD of 22% ($P<0.001$) during six months of bottle storage. ACE maceration applied on the first day of fermentation resulted in wines at six months bottle age that had 38% ($P<0.001$) greater WCD than Control wines. The addition of pectolytic enzymes made no significant difference to WCD (Table 7a.4).

Hue: While there was no significant effect on wine hue imposed by ACE maceration or seed removal relative to crushed berry Control wines, the addition of pectolytic enzyme resulted in a lower wine hue (average 8%;$P<0.001$) at bottling (see Appendix 7a.2). At six months bottle age, the average hue of wines with no pectolytic enzymes was 14% ($P=0.027$) higher than in the Control wines, whereas the addition of pectolytic enzymes mitigated the increase to 10%. Only when ACE maceration was applied on day 5 of fermentation, was there no effect of pectolytic enzyme on wine hue.

Hue SO_2: From bottling to six months bottle age, hue SO_2 (stable) values decreased by an average of 23% for all treatments ($P<0.001$). Application of ACE maceration on day 1 of fermentation reduced the value of SO_2 by 18% ($P<0.001$) relative to the crushed berry Control wines (Table 7a.4). By contrast, the addition of pectolytic enzyme resulted in an average reduction in hue SO_2 of only 6% ($P<0.001$) (data not shown).

In summary, experiment C results showed that ACE maceration resulted in wine that was higher in tannin, had more stable pigment, greater colour density and more intense blue-purple colouration of the stable pigment fraction than did crushed berry Control wines, those made following seed removal or those fermented with pectolytic enzymes.

7a.5 DISCUSSION

In this study an innovative mechanical maceration technique (ACE maceration) was introduced that reduced grape skin particle size during fermentation. The technique was shown to have a significant impact on tannin, stable pigment, wine colour density and hue of Pinot Noir wines, that was far greater than the effect of pectolytic enzymes. The application of ACE maceration on day one of fermentation was more effective than application of the technique later in the fermentation process.

According to published literature this is the first study that examines the effect on wine phenolic composition of mechanically reducing the skin particle size of the pomace while causing minimal damage to the seeds. The treatment released sufficient colour pigments and tannin to produce wines that were more intensely coloured with greater blue-purple colouration. The changes in phenolic attributes were consistent with putative improvements in Pinot Noir wine quality.

The grapes of Clone G5V15 (experiments A and B, 2011) were 40% lower in both anthocyanin and tannin than were the grapes of Clone 115 (experiment C, 2012) however both clones had a tannin to anthocyanin ratio of 12 to 1. This is in sharp contrast to reported ratios of anthocyanin to tannin in Cabernet Sauvignon and Shiraz grapes, being 2.1 to 1 (Holt et al., 2008) and 1.6 to 1 (Ristic et al., 2010) respectively and highlights the uniqueness of Pinot Noir in balancing the extraction of desirable phenolic compounds from grapes into wine.

The grapes of Pinot Noir Clone 115 used in Experiment C were 66% higher in tannin than grapes from Clone G5V15 (Experiments A and B) (Table 7a.1). At 6 months bottle age, the tannin concentration of wines made from grapes of Pinot Noir Clone G5V15 was 4.5-fold higher in wine tannin and 46% higher in NBP than the crushed berry control wines (Tables

7a.2 and 7a.3). Conversely, wines made from Clone 115 grapes (experiment C) were 3-fold higher in tannin and 36% higher in NBP than their crushed berry counterparts. When comparing the relative increases between control wines and those with cut skins for each of the three experiments, a higher concentration of wine tannin and lower concentration of NBP was observed in wines made from grapes with a lower initial concentration of grape tannin. This suggested that the formation of non-bleachable pigment in the wine (derived from anthocyanin-tannin adducts) was limited by the tissue source of the tannin. The wine making experiments in this study together with experiments described in Chapters 4 and 6 point to the tannin source as the major limitation, with excess seed tannins found to limit the formation of non-bleachable pigment in Pinot Noir wines (Sparrow et al., 2012b, Sparrow et al., 2014).

Berry composition data provides valuable information about the total phenolics available in the grape, however as this study confirmed, the phenolic status of the wine is determined by differences in extractability of phenolic components from skin and seed tissues during winemaking (Vidal et al., 2004c, Cortell et al., 2005, Koyama et al., 2007a).

The more rapid release of phenolic components from ACE macerated musts was attributed to the 16-fold increase in broken skin edges. When applied in conjunction with pectolytic enzyme addition (Experiment C), ACE maceration resulted in significantly higher tannin concentrations however no further difference was observed in either NBP or wine colour density (see Appendix 7a.2). In studies of pectolytic enzyme effects on grape tissues, Meyer et al. (1998) showed that milling freeze-dried grape pomace reduced particle size to surface area of 15 to 62 mm^2, ACE macerated particles fell within this range (22.4 mm^2). When milling was followed by extraction in the presence of pectolytic enzymes, these researchers observed a positive effect on the recovery of phenols from grape pomace which they

attributed to an increase in enzyme catalysed polysaccharide hydrolysis of cell wall material of the undifferentiated grape tissues, rather than to reduced particle size *per se*. The experiment described here demonstrated that ACE maceration had such a positive impact on colour extraction from the grape skins that the effect of pectolytic enzyme was secondary. Watson et al. (2000) reported a 38% increase in tannin concentration in Pinot Noir wines macerated in the presence of pectinase enzymes, and attributed 70% of this increase to the extraction of seed tannin.

The grape musts for experiment A were prepared with high concentrations of pectinase enzyme specifically to optimise seed release from the pomace so that they could be removed early in fermentation. However seed removal had little impact on most of the phenolic parameters measured, with the exception of WCD, which decreased when seed removal was combined with pectinase addition and a reduction in skin particle size.

The seed to skin tannin ratio of Pinot Noir grapes is high relative to other red wine grape varieties (Mattivi et al., 2009) with grape seed tannins outweighing skin tannins by 2.9 to 1 (Cortell et al., 2005, Cortell and Kennedy, 2006, Kemp et al., 2011) which has a significant bearing on the formation of NBP in Pinot Noir wines (Ristic et al., 2010). The choice of maceration technique largely determines the ratio of skin to seed tannin in the wine. The effect of grape pulp tissues on seed tannin extraction (Chapter 4) indicates that the proportion of seeds that are released from the pulp tissues of the floating pomace cap during fermentation, together with the gradual loss of integrity of the seed tissues, has a significant bearing on the seed tannin concentration of the wine matrix. The experiments described here demonstrated that reducing the particle size of the pomace components increased tannin by 5 to 6-fold. Although sensory properties were not examined in this

study, such high tannin concentrations have implications for the perception of wine astringency (McRae et al., 2010, McRae and Kennedy, 2011).

The high tannin concentration observed when skin and seed particles were homogenised simultaneously was not, however, reflected in an enhanced development of NBP, suggesting that the additional seed tannin released was not involved in the formation of NBP. As wine subjected to this treatment still contained a significant concentration of free anthocyanin after six months bottle age, an equilibrium effect between free anthocyanins and anthocyanin-tannin adducts may be implicated. Alternatively, the nature of the seed tannins may have limited the formation of NBP. The low anthocyanin concentration observed in ACE wines supports the latter alternative as do experiments described previously (Sparrow et al., 2014) leading to the conclusion that skin tannin was primarily involved in the formation of pigmented polymers.

ACE maceration applied on the first day of the fermentation was apparently more effective than its application later in the fermentation period. In support of these observations, Gambuti et al. (2009) found that the maximum release of flavan-3-ols (tannin precursors) from the grape skins of red wine varieties Aglianico and Uva di Troia, was achieved on the first day of maceration. These researchers also noted that maceration time (pomace contact) had a greater influence on the extraction of seed tannin than did the alcohol concentration and attributed this to the solubility of the seed coat.

In their work with Shiraz grapes, Ristic et al. (2010) proposed that a valuable indicator of wine quality determined by a sensory panel, could be obtained by multiplying the anthocyanin concentration of the wine by the ratio between skin and seed tannin, with a higher score being an indication of superior wine quality. Studies with Pinot Noir described

in Chapter 6 showed that this wine quality score had a significant correlation with both the intensity and colour stability of the wine.

7a.6 CONCLUSION

The ACE maceration technique is likely to provide the greatest phenolic quality benefits to red wine varieties that have unusual polyphenolic profiles such as Pinot Noir, Nebbiolo, Gamay, Sangiovese and Barbera. ACE has the potential to replace pre-fermentation (cold soaking) and post fermentation (extended maceration) treatments currently employed for lightly pigmented red wine varieties. ACE maceration may not only provide winemakers with the option of promoting the extraction of skin polyphenols relative to seed polyphenols, without the removal of seeds, but may have broader application in the vinification of other red wine varieties, as the early development of stable wine polyphenols provides the opportunity for early press off from the grape pomace, thereby increasing winery throughput.

Appendix 7a.1 Treatment means (n=4) of Pinot Noir wine phenolic parameters for five maceration treatments at bottling and 6 months bottle age.

Phenolic parameter	Anthocyanin (g/L)	Tannin (g/L)	†NBP (AU)	‡WCD (AU)	⁄Hue	⁋Hue SO_2
	Wine at bottling (50 days post-inoculation)					
crushed berries (control)	0.13	0.38 c	0.25 b	2.02 b	0.75 b	1.85 a
remove seeds	0.12	1.72 b	0.40 a	2.67 a	0.77 a	1.84 a
homogenise skins	0.12	0.24 c	0.22 b	1.74 b	0.77 a	1.50 b
homogenise skins, remove seeds	0.12	1.90 b	0.37 a	2.48 a	0.75 b	1.58 b
homogenise skins and seeds	0.11	2.41 a	0.42 a	2.68 a	0.75 b	1.53 b
	Wine at 6 months bottle age (230 days post-inoculation)					
crushed berries (control)	0.09 a	0.45 c	0.48 c	2.60 c	0.79	1.38 a
remove seeds	0.08 a	0.27 c	0.38 c	2.09 d	0.80	1.49 a
homogenise skins	0.08 a	1.93 b	0.68 ab	3.30 ab	0.79	1.22 ab
homogenise skins, remove seeds	0.07 ab	1.99 b	0.62 b	3.03 b	0.82	1.31 ab
homogenise skins and seeds	0.06 b	2.66 a	0.73 a	3.46 a	0.78	1.19 b
¥LSD (trt x time)	0.02	0.34	0.11	0.38	NS	0.18
p-value	*0.009*	*<.001*	*<.001*	*<.001*	*0.646*	*0.021*

†Non-bleachable pigment; ‡Wine Colour Density; ⁄Hue (ratio A420:A520 in model wine solution) ⁋Hue SO_2 (ratio A420:A520 in model wine solution and 0.375% sodium metabisulphite); ¥Least Significant Difference. For each time period, numbers within a column with different letters are significantly different at $P \leq 0.05$. NS, Not significant at $P \leq 0.05$.

Appendix 7a.2 Pinot Noir wine phenolic parameters at 50 (bottling) and 230 days (6 months) post-inoculation (Mean; n=4) for seven maceration treatments in the presence or absence of pectolytic enzymes.

Days post inoculation	Enzyme status	control	cut cap day 1	cut cap day 3	cut cap day 5	cut cap and remove seeds day 1	cut cap and remove seeds day 3	cut cap and remove seeds day 5	P-value	¥LSD (trt)	¥LSD (trt x time)
						Anthocyanin (g/L)					
50	none	0.31	0.28	0.29	0.30	0.26	0.26	0.28	0.04	0.04	NA
230	none	0.24	0.20	0.20	0.21	0.19	0.18	0.18	<0.001	0.25	0.03
50	plus	0.31	0.27	0.26	0.27	0.28	0.28	0.29	<0.001	0.03	NA
230	plus	0.26	0.17	0.18	0.19	0.20	0.19	0.21	0.01	0.26	0.35
						Tannin (g/L)					
50	none	0.79	3.05	2.75	2.71	2.83	2.64	2.63	<.001	0.36	NA
230	none	0.91	3.24	2.85	2.86	3.04	2.83	2.54	0.01	0.23	0.24
50	plus	1.08	3.27	3.33	3.05	3.28	3.19	2.97	<0.001	0.29	NA
230	plus	1.18	3.52	3.56	3.28	3.54	3.43	3.23	<0.001	0.27	0.35
						Non-bleachable pigment (AU)					
50	none	0.56	0.97	0.84	0.56	0.85	0.77	0.67	<.001	0.20	NA
230	none	1.10	1.54	1.31	1.44	1.43	1.32	1.28	0.38	NS	0.23
50	plus	0.49	1.03	0.74	0.73	0.87	0.86	0.77	<0.001	0.17	NA
230	plus	0.92	2.39	1.22	1.29	1.51	1.57	1.33	<0.001	0.31	0.42
						Colour density (AU)					
50	none	4.54	6.69	6.10	6.09	6.07	5.52	5.05	<0.001	0.97	NA
230	none	5.82	7.64	6.71	7.21	7.09	7.13	6.59	0.21	NS	0.90
50	plus	4.19	7.01	5.59	5.71	6.54	6.32	5.95	<0.001	0.84	NA
230	plus	5.34	8.99	7.20	6.61	7.65	7.67	7.07	<0.001	0.97	1.26

¥Least Significant Difference. For each time period, numbers within a column with different letters are significantly different at P ≤ 0.05. NA, Not applicable; NS, Not significant at P ≤ 0.05.

7a.7 SUBSEQUENT RESEARCH FOCUS

Significant improvements in the phenolic composition of Pinot Noir wines made by reducing the skin particle size of the grape pomace have been demonstrated. Consequently there is clear benefit in evaluating the sensory properties of ACE macerated wines. Changes in quality parameters such as appearance, aroma, taste and palatability are likely to be manifested by the changes reported in the wine phenolic parameters determined by spectroscopy.

7B CUTTING EDGE PINOT NOIR: INNOVATIVE MACERATION TECHNIQUE EFFECTS THE PHENOLIC, SENSORY AND AROMA CHARACTERISTICS OF PINOT NOIR WINES

This Chapter is in preparation for submission to a scientific journal

Title: Cutting Edge Pinot Noir: Innovative ACE maceration technique affects the phenolic, sensory and aroma characteristics of Pinot Noir wines

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The authors acknowledge the wine aroma analysis overseen by Natoiya Lloyd from the Australian Wine Research Institute

7b.1 ABSTRACT

Earlier work (Chapter 7a) demonstrated the significant impact on the phenolic components of Pinot Noir wines observed in response to reducing the grape skin particle size (ACE maceration). This investigation evaluated the phenolic composition and sensory profiles of Pinot Noir wines fermented with four different micro-vinification techniques that modified the floating pomace cap during fermentation: (1) Plunge, (2) Submerge, (3) reduced skin particle size (ACE), and (4) ACE combined with Submerge. Wines were analysed for anthocyanins, tannins, non-bleachable pigments, colour density, hue and volatile aroma constituents. Sensory descriptive analysis was also conducted using 12 judges to evaluate colour, aroma, flavour and overall wine quality. Wines from the ACE vinification were found to have the highest proportion of red colour, tannin, non-bleachable pigments, fruit and floral aromas. Wines from the Submerged Cap vinification had more dark cherry aromas and flavour. Increased ester concentration (mainly acetates) and total volatile compounds were found in wines from the ACE maceration compared with either the conventional plunged cap or the Submerged Cap maceration. Principal component analysis distinguished three major attributes of wines made with reduced particle size, based on appearance,

aroma and palate. Linear regression analyses revealed strong correlations between sensory and instrumental phenolic measurements relating to several descriptive wine parameters: red colouration and both colour density (r^2=0.95) and non-bleachable pigment (r^2=0.95); dark fruit flavour and both colour density (r^2=0.85) and non-bleachable pigment (r^2=0.85); astringency and both tannin (r^2=0.97) and non-bleachable pigment (r^2=0.87). The results of the study demonstrated that increasing the perimeter of total skin edges early during vinification allowed for the rapid release of anthocyanins and skin tannins which in turn polymerised to form non-bleachable pigments accounting for significant changes in the sensory attributes of the wine.

7b.2 INTRODUCTION

The pigment content of *Vitis vinifera* cv. Pinot Noir grapes is low compared with other red grape varieties (Wenzel et al., 1987). In addition they contain no acylated anthocyanins making them vulnerable to ageing, the colour changing from red to tawny-brown. These characteristics of Pinot Noir make it well suited to a wine style that is usually consumed young (Sacchi et al., 2005), and carbonic maceration (Flanzy, 1935) or minimal grape crushing is often used in the preparation of Pinot Noir wines to enhance the fruit and floral characteristics inherent in the variety. However, mild grape crushing techniques cause minimal disruption to the berry tissues consequently compromising colour and tannin extraction from the grapes (Pinelo et al., 2006). In their study with Merlot grapes Cerpa-Calderón and Kennedy (2008) increased the proportion of crushed grapes in must preparations by 25% increments and found that the greatest amount of skin tannin was extracted when 75% of the fruit was crushed, and that the extraction rate for skin tannin was faster than that of seed tannins. An earlier review (Sacchi et al., 2005) compared a

range of maceration techniques used to enhance colour and tannin extraction in lightly pigmented grape varieties. However no single method of vinification has been found to optimise the distinctive characteristics of Pinot Noir wine. In a more recent review of anthocyanin derived pigments and colour evolution of red wines, He et al. (2012a) concluded that further research was need to provide new oenological practices to improve anthocyanin stability, formation of stable pigments, and total wine colour.

In previous studies described in this thesis, it was observed that reducing the skin particle size of the floating pomace cap in the early stages of fermentation, had a significant impact on both the tannin and non-bleachable pigment concentration of Pinot Noir wines (Sparrow et al., 2013a) as did Submerged Cap fermentation (Dambergs and Sparrow, 2011). In order to make the transition from research trials to commercial trials, ACE maceration was applied in combination with alternative cap management techniques: 1. 'twice daily plunging' which is a conventional method of cap management for the majority of red wine varieties during which the floating pomace cap is physically plunged (often by hand) to maximise skin contact with the fermenting juice; and 2. 'Submerged Cap' (in which a physical barrier is inserted into the fermentation vessel to prevent the pomace cap from floating to the surface, thereby keeping all of the grape skins in constant contact with the fermenting juice (as used in experiments described in Chapter 7a). This study evaluated the phenolic composition, sensory and aroma properties of Pinot Noir wines at six months bottle age, made using these vinification techniques.

7b.3 MATERIALS AND METHODS

7b.3.1 Grape sampling and analysis:

Grapes of *Vitis vinifera* cv. Pinot Noir clone D5V12, from drip irrigated vines, trained to vertical shoot positioning, were harvested from a 14-year old vineyard in northern Tasmania in April 2013. Fruit was hand harvested and bunches randomly allocated to four 12 kg replicates. Prior to fermentation, 100 berries were selected at random to determine fruit composition. The berries were hand-crushed and the juice drained for fruit composition analyses. Total soluble solids (°Brix) were measured using a hand-held refractometer, the pH of the juice was measured using a Metrohm pH meter/autotitrator and titratable acidity was determined by titration with 0.333 M NaOH to an end point of pH 8.2 and reported as g/L tartaric acid. A further 200 g of berries from each replicate were frozen at 20 ^{o}C for later analysis of grape colour (Iland et al., 2004) and tannin (Dambergs et al., 2012b).

7b.3.2 Treatments for wine-making:

Grapes from each of the four replicates were de-stemmed and crushed using a Marchisio Grape Crusher/Destemmer (1000 to 1500 kg/h) to produce 10 kg of must, which was placed in a 20 L food grade plastic bucket. In addition to reducing the skin particle size, two maceration techniques were investigated in this study: twice daily plunging and Submerged Cap micro-vinification. Twice daily plunging was effected by hand using a broad wooden paddle while Submerged Cap vinification was effected by placing a slotted nylon plate, 260 mm in diameter, on top of the floating cap in the 20 L food grade plastic buckets. Sufficient downward pressure was applied such that the pomace cap was slightly submerged and the nylon plate was held in place by a stainless steel wire frame attached to the walls of the fermentation vessel. In treatments where the skin particle size was reduced, the floating

pomace cap of the ferment was cut into small fragments 24 hours after inoculation, using a Robot Coupe Stick/Immersion Blender, CMP 350 V.V. (350 mm shaft length). This device allowed the skins to be cut into fragments that were approximately 4% of their original size whilst dispersing the seeds through gaps in the blade housing, ensuring that seeds did not come into contact with the cutting blades. Treatments for this trial were: (1) plunge twice daily (Control); (2) Submerge Cap; (3) ACE, plunge twice daily (4) ACE and Submerge Cap.

7b.3.3 Vinification protocol

After preparing the musts, 50 mg/L SO_2 was added to each fermentation vessel in the form of potassium metabisulphite. Six hours later the must preparations had equilibrated to ambient temperature and were inoculated with 300 mg/L RC212 yeast solution and fermented under Controlled temperature conditions at 27°C (±1°C). All fermentation vessels were covered with a loose fitting lid to allow adequate gas exchange. Approximately 24 hours after inoculation, the pomace cap had floated to the surface of each fermentation vessel and the cap cutting procedure was applied to treatments 3 and 4 and the Submerged Cap apparatus was inserted into fermentation vessels for treatments 2 and 4. On day three of the fermentation, 300 mg/L of diammonium phosphate was added to each fermentation vessel. After ten days, each fermenting must had proceeded to dryness which was confirmed at less than 2 g/L of residual sugar using Clinitest® reagent tablets (Bayer Australia Ltd.). Each ferment replicate was then individually pressed in a flat-bed press at 200 Kpa of pressure to recover 6.0 L of wine which represented 60% (v/w) of the must weight.

7b.3.4 Quantifying pomace cap maceration on wine phenolics

Samples were clarified by centrifugation at 5,000 rpm for five minutes using an Eppendorf 5417C centrifuge with an Eppendorf F45-30-11 rotor (Crown Scientific, Australia). The

phenolic composition of wine samples was determined using the modification of the Somers assay described by Mercurio et al. (2007) and rapid tannin analysis (Dambergs et al., 2012b). Wine phenolic parameters calculated from these measures were: free anthocyanin, non-bleachable pigment, colour density, hue and hue SO_2 (hue of non-bleachable pigments).

7b.3.5 Statistical analysis for chemical composition*:*

Mean, standard deviation and co-efficient of variation were calculated for fruit composition parameters. Statistical analyses were conducted using GenStat 64-bit Release 16.1 Copyright 2013, VSN International Ltd. Data for individual phenolic components in wine samples were analysed using Repeated Measures ANOVA, with treatment as the main plot and days from inoculation as the repeated measure. For each of the phenolic parameters assessed, ANOVA was followed by post-hoc analysis using Fisher's Protected Least Significant Difference (LSD) test. Linear Regression analyses (Microsoft® Excel 2007) were conducted to assess the correlation between phenolic parameters and sensory properties of the wine ($P \leq 0.05$).

7b.3.6 Aroma analysis by Gas Chromatography-Mass Spectrometry

The aroma analysis was performed by Metabolomics Australia –South Australian Metabolmics Facility at the Australian Wine Research Institute (AWRI) and was conducted as a profiling experiment (qualitative) which enabled the comparison of treatment effects on the overall profile of important volatile fermentation products in wine. The internal standards ethyl acetate, ethyl propanoate, ethyl 2-methylpropanoate, ethyl butanoate, ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, 2-methylpropyl acetate, 2-methylbutyl acetate, 3-methylbutyl acetate, 2-methylpropanol, butanol, 2-methylbutanol, 3-methylbutanol, ethyl hexanoate, hexyl acetate, hexanol, ethyl decanoate were supplied by Sigma-Aldrich. The

internal standards were used to correct for matrix effects and instrument variability. Labelled standards purchased from Sigma-Aldrich were d8-ethyl acetate, d10-butanol, d13-hexanol. Each had >98 atom % deuterium. The corresponding deuterated internal standard for each compound was used to calculate response ratio of each compound relative to the standard sample. Analytical performance and method validation details were conducted as described by (Siebert et al., 2005).

All solvents for aroma analyses were Mallinckrodt nanopure grade. The solvents and analytical standards were verified for purity by GC-MS prior to use. The analysis of wine volatiles for each treatment replicate was performed on an Agilent 7890 gas chromatograph equipped with Gerstel MPS2 multi-purpose autosampler and coupled to an Agilent 5975C XL mass selective detector. The gas chromatograph was fitted with a 30 m x 0.18 mm Restek Stabilwax – DA (crossbond carbowax polyethyleneglycol) of 0.18 mm film thickness with a 5 m x 0.18 mm retention gap. Helium was used as the carrier gas with flow-rate 0.6 mL/min in constant flow mode. The oven temperature began at 35^{o}C, was held at this temperature for 4 minutes, then heated to 60^{o}C at 4^{o}C per minute, further heated to 100^{o}C at 16^{o}C per minute, then heated to 230^{o}C at 8^{o}C per minute and held at this temperature for five minutes. The volatile compounds were isolated using large volume headspace sampling and injected into a Gerstel PVT (CIS 4) inlet fitted with a Tenax TA liner. The injector was heated to 330^{o}C at 12^{o}C per minute. Positive ion electron impact spectra at 70eV were recorded in scan mode. Wine samples (in triplicate) were diluted (2:5) in buffer solution (10% (w/v) potassium hydrogen tartrate, pH adjusted with tartaric acid to 3.4. A total of 16 authentic volatile compounds were analysed concurrently with the wine samples and each sample was spiked with deuterated internal standard.

A pooled 'Master mix' sample was prepared by taking an equal volume of aliquot from each sample and mixed well. It was treated and diluted in the same way as the wine sample. It was injected after every 5 sample as a quality control check to monitor the instrument's performance during a sequence.

7b.3.7 Sensory evaluation

Training: A panel of twelve assessors trained in descriptive analysis by the Australian Wine Research Institute was convened for this study. Panellists attended three training sessions to determine appropriate descriptors for rating the wines in the formal sessions. During these sessions the panellists assessed wines from the study, which represented the full range of sensory properties that were assessed by appearance, aroma and palate to generate and refine terms appropriate to the wines under study. In sessions two and three, standards for aroma attributes were presented, which were made available during the booth practice session.

Following the three training sessions, tasters participated in a practice session in the sensory booths under the same conditions as those for the formal sessions. After the practice session, any terms which needed adjustment were discussed and the final list of attributes determined. For the formal session, a final list of attributes was refined to include one appearance, nine aroma and thirteen palate attributes. These attributes definitions and synonyms and where appropriate, the standards provided, are shown in Table 7b.1.

Table 7b.1: Attributes, definitions and standards evaluated by tasters in formal sessions.

Attribute	Definition/Synonyms	Standard
Appearance		
Red	Intensity of the colour red in the sample	
Aroma		
Overall fruit intensity	Intensity of the fruit aromas in the sample	
Red fruits	Intensity of the aroma of red fruits and berries: raspberries, strawberries, cherries, cranberries	3 x frozen raspberry, 3 x frozen cranberry (Sara Lee brand)
Dark fruits	Intensity of the aroma of dark fruits and berries: blackberries, plums, black currants, mulberries	2 x frozen blackberry, 2 x frozen blueberry (Sara Lee brand)
Confection	Intensity of the aroma of confection and lollies, musk, Turkish delight	1 x red raspberry lolly, 1 x red snake (Natural Confectionary Company), no wine
Stalky	Intensity of the aroma of stalks, leaves, rhubarb	1 pc tomato stalk, 2 cm long; 4 pc rhubarb (1 cm cubes), no wine
Earthy	Intensity of the aroma of wet earth, organic matter	20 µL of 4 mg/L Geosmin
Medicinal	Intensity of the aroma of medicine, hospitals, bandaids	Isopropyl alcohol wipe; no wine
Sweaty/Cheesy	Intensity of the aromas of sweat, body odour, cheese	50 µL of 10.5 g/L Hexanoic acid, 20µL 11.9 g/L Isovaleric acid
Drain	Intensity of the aroma of drains, rotting organic matter	1 tsp tinned asparagus; 1/16 tsp. ash
Pungent	Intensity of the aroma and prickly effect of alcohol	4 mL ethanol
Other	Intensity of any other aroma in the sample	

Palate	
Overall flavour intensity	Intensity of fruit flavours in the sample
Red fruits	Intensity of the flavour of red fruits and berries: raspberries, strawberries, cherries, cranberries
Dark Fruits	Intensity of the flavour of various dark fruits: blackberries, currants, plums
Stalky	Intensity of the flavour of green stalks, green leaves, rhubarb
Viscosity	The perception of the body, weight or thickness of the wine in the mouth. Low = watery, thin mouth feel. High = oily, thick mouth feel.
Astringency	The drying and mouth-puckering sensation in the mouth. Low = coating teeth; Medium = mouth coating & drying; High = puckering, lasting astringency.
Bitter	The intensity of bitter taste perceived in the mouth, including after expectorating
Hotness	The intensity of alcohol hotness perceived in the mouth, Low = warm; High = hot.
Fruit Aftertaste	Intensity of the lingering fruit flavour perceived in the mouth after expectorating
Burning Aftertaste	Intensity of the lingering burning sensation in the mouth after expectorating
Other	Intensity of any other attribute on the palate

All aroma standards were added to 30 mL of 2012 Yalumba Classic Dry Red unless otherwise noted.

Sensory assessment: Samples were presented to panellists in 30 mL aliquots in 3-digit-coded, covered, ISO standard wine glasses at 22 to 24°C, in isolated booths under daylight lighting, with randomised presentation order. The assessors were presented with four trays of four wines per tray with a two minute rest between samples and a ten minute break between trays. Samples were evaluated over two days of formal sessions. Wines were presented to assessors twice, 32 samples in total.

Statistical analysis for sensory evaluation: The intensity of each attribute was rated using an unstructured 150 mm line scale, with indented anchor points of 'low' and 'high' placed at 10% and 90% respectively. Data were acquired and converted to scores between 0 and 10, using Fizz sensory software (version 2.46, Biosystemes, Couternon, France). Panel performance was assessed using Fizz, Senstools (OP&P, The Netherlands) and PanelCheck (Matforsk) software, and included analysis of variance for the effect of sample, judge and presentation replicate and their interactions, degree of agreement with the panel mean and degree of discrimination across samples. All judges were found to be performing to an acceptable standard.

Analysis of variance (ANOVA) was carried out using JMP 5.0.1a (SAS Institute, Cary, NC). The effects of treatment and judge were assessed, with treatment by judge interaction as a random effect in the ANOVA. Following ANOVA, Fisher's least significant difference (LSD) value was calculated ($P<0.05$). Principal component analysis (PCA) was conducted on the mean values averaged over panellists and replicates, using the correlation matrix using Fizz and The Unscrambler 10.1 (CAMO Software AS, Oslo, Norway).

7b.4 RESULTS

7b.4.1 Fruit composition

The berry composition and phenolic content of grapes (*Vitis vinifera* cv. Pinot Noir grapes clone D5V12) used in this study are listed in Table 7b.2. The ratio of tannin to anthocyanin in the fruit was 12:1 as noted for other clones of Pinot Noir (Chapter 7a). The tannin and anthocyanin concentration for the grape samples were within the range reported in literature for Pinot Noir (Cortell and Kennedy 2006; Cortell et al. 2005; Cortell et al. 2007; Kemp et al., 2011).

Table 7b.2 Composition of fresh Pinot Noir grapes (mean ± standard deviation, n=4).

Berry compositional parameter		
Berry weight (g FW‡)	1.37	± 0.13
Total Soluble Solids (°Brix juice)	21.20	± 0.49
Juice pH	3.22	± 0.04
Titratable acidity (g/L juice)	7.70	± 0.24
Anthocyanin (mg/g FW‡ berry)	0.58	± 0.03
Total tannin (mg/g FW‡berry)	6.87	± 0.24

‡FW, fresh weight

7b.4.2 Phenolic composition of wine

A number of significant interactions between treatment and time were observed in this study (Table 7b.3), those with the greatest potential for commercial application of the technique were selected for detailed interpretation.

Anthocyanin: Analysis at six month bottle age identified that ACE wines alone, had significantly higher anthocyanin concentrations (0.25 g/L) than the Control wines (0.16 g/L).

Tannin: The reduction of skin particle size in ACE wines resulted in an average increase in tannin concentration of 6.3-fold compared with the Control wine concentration of 0.32 g/L (Table 7b.3). As early as 3 days post inoculation, the tannin content of ferments in which the pomace cap had been cut were 3.6-fold higher than the Control wines, while submerging the ACE resulted in a 5-fold increase in tannin. By six months bottle age, ACE wines were 6.8-fold higher than Control wines (0.31 g/L) and 'ACE and Submerged Cap' wines were 7.6-fold higher in tannin than the Control wines.

Table 7b.3. Changes in phenolic parameters of Pinot Noir wine from inoculation to six months bottle age, comparing ACE macerated and Submerged Cap vinification treatments.

Days post inoculation	1	2	3	5	7	8	10	50	230	*LSD (trt x time)*	*P-value*
Tannin concentration (g/L)											
Control	0.00 b	0.22 b	0.39 c	0.28 c	0.43.b	0.43 d	0.35 c	0.33 c	0.31 c		
Submerge	0.00 b	0.24 b	0.51 c	0.57 c	0.73 b	0.78 c	0.70 b	0.63 c	0.78 b	*0.35*	*<0.001*
ACE	0.52 a	1.24a	1.39 b	1.71 b	1.85 a	1.92 b	2.05 a	1.89 b	2.14 a		
ACE & Submerge	0.21 b	1.18 a	1.95 a	2.23 a	2.13 a	2.34 a	2.28 a	2.31 a	2.37 a		
Anthocyanin concentration (g/L)											
Control	0.16 b	0.22 b	0.28	0.22 b	0.21 b	0.21 b	0.18	0.19	0.15 b		
Submerge	0.15 b	0.23 ab	0.29	0.27 ab	0.28 a	0.28 a	0.25	0.24	0.23 a	*0.06*	*<0.001*
ACE	0.25 a	0.30 a	0.29	0.25 ab	0.22 b	0.22 b	0.22	0.21	0.18 ab		
ACE & Submerge	0.18 b	0.28 a	0.32	0.28 a	0.26 ab	0.27 ab	0.25	0.24	0.17 b		
Non-bleachable pigment (AU)											
Control	0.20 c	0.26 b	0.26 b	0.26 b	0.32 ab	0.31 b	0.33 c	0.32 b	0.49 c		
Submerge	0.17 c	0.24 b	0.28 b	0.28 b	0.30 ab	0.34 b	0.44 b	0.38 b	0.61 b	*0.10*	*<0.001*
ACE	0.50 a	0.45 a	0.45 a	0.46 a	0.47 a	0.56 a	0.61 a	0.66 a	0.93 a		
ACE & Submerge	0.30 b	0.39 a	0.41 a	0.44 a	0.39 a	0.49 a	0.54 a	0.58 a	0.90 a		

Colour Density (AU)											
Control	2.45 c	2.81 b	2.64 b	2.46 b	2.61 c	2.76 c	2.83 b	2.62 c	2.96 c		
Submerge	2.41 bc	2.72 b	3.01 b	2.97 b	3.05 bc	3.34 b	3.40 b	3.41 b	4.09 ab	*0.68*	*0.005*
ACE	4.25 a	4.34 a	3.98 a	3.93 a	3.75 a	4.35 a	4.55 a	4.54 a	4.97 a		
ACE & Submerge	3.03 b	3.92 a	4.03 a	3.98 a	3.43 ab	4.16 a	4.29 a	4.34 a	4.68 a		
Hue (AU)											
Control	0.91 b	0.71 b	0.67 b	0.70 b	0.74 a	0.74 a	0.74 a	0.68 ab	0.75 b		
Submerge	0.93 ab	0.69 b	0.65 b	0.69 b	0.69 b	0.69 b	0.69 b	0.66 b	0.70 c	*NS*	*0.06*
ACE	0.98 a	0.74 a	0.75 a	0.75 a	0.77 a	0.77 a	0.77 a	0.72 a	0.79 a		
ACE & Submerge	0.97 a	0.70 ab	0.69 b	0.68 b	0.68 b	0.68 b	0.67 b	0.65 b	0.73 bc		
^{ħ}Hue SO_2 (AU)											
Control	2.99 a	2.06	1.96 b	1.90 bc	1.93 a	1.90 a	1.80 a	1.67 a	1.44 a		
Submerge	3.08 a	2.09	2.02 b	2.03 a	1.98 a	1.96 a	1.76 ab	1.72 a	1.46 a	*0.1*	*<0.001*
ACE	2.55 b	2.04	2.03 b	1.89 bc	1.84 b	1.77 b	1.68 b	1.53 b	1.34 b		
ACE & Submerge	2.76 b	2.03	2.94 a	1.81 c	1.74 b	1.64 c	1.55 c	1.42 c	1.20 c		

Mean (n=4) phenolic characteristics of Pinot Noir wine from four maceration treatments: 1. Control; 2. Submerge Cap; 3. ACE; 4. ACE and Submerge, at six months bottle age (230 days post-inoculation) determined by UV-VIS spectroscopy and chemometrics. Data analysed by Repeated Measures ANOVA followed by Fisher's Least Significant Difference (LSD) Test. ^{ħ}Hue resistant to sulphur dioxide bleaching. Results in the same column for each phenolic parameter having different letters, are significantly different at $P<0.05$. NS, Not significant at $P \leq 0.05$.

Non-bleachable pigment (NBP): ACE maceration resulted in an average increase of 75% in NBP relative to the Control value of 0.31 AU (Table 7b.3). From day 1 the increase in NBP of 'ACE wines' was 2.5-fold greater than in Control wines. Submerging the cap apparently mitigated the effect of applying ACE early in fermentation (37% higher NBP than Control on day 1) but by six months bottle age there was no significant difference in NBP between ACE wines and ACE and Submerge Cap wines.

Wine colour density (WCD): From day 1 the wine colour density for ACE treated ferments was 73% ($P<0.001$) higher than the Control wines (Table 3). At six months bottle age, 'ACE wines' remained 68% ($P<0.001$) higher than the Control wines and 22% higher than Submerged Cap wines. ACE maceration in combination with submerging the cap made no significant difference to WCD compared with ACE maceration alone.

Hue: The hue of all wines increased by an average of 10% ($P<0.001$) from bottling to six 6 months bottle age. Submerging the pomace cap resulted in a significant reduction (6%; $P<0.001$) in the hue of six month old wines compared to the Control wines (Table 7b. 3). When the pomace cap was treated with ACE and Submerge cap, the hue was only 3% ($P<0.001$) lower than the Control wines, while ACE maceration alone increased the wine hue by 5% ($P<0.001$). The lower wine hue observed for the 'Submerged Cap' treatments was indicative of a greater proportion of blue-purple and less red-brown colouration in the wine than that associated with the conventional 'Control' Pinot Noir ferments.

Hue SO_2: From day seven of fermentation both ACE maceration treatments were significantly lower in hue SO_2 (9%; $P<0.001$) than the treatments where berries were only crushed (Control and Submerged Cap treatments) (Table 7b.3). However by six months bottle age, wines made from ACE macerated ferments but which were not Submerged, were 8% ($P<0.001$) lower in hue SO_2, and those that combined ACE maceration with

Submerged Cap were 20% lower in hue SO_2. Lower wine hue SO_2 values were indicative of a greater proportion of stable pigments in the wine that had blue-purple colouration as opposed to yellow-orange.

In summary, the combination of ACE maceration and Submerged Cap management resulted in wines that were higher in tannin, had greater colour density, a greater concentration of stable non-bleachable pigments, and a larger proportion of stable blue-purple pigments relative to the conventionally treated Control wines that had been plunged twice daily.

7b.4.3 Wine aroma analyses

Significant differences were observed between the Control wines and those made from must treated with either ACE maceration or Submerged Caps (Table 7b.4). While submerging the cap resulted in a significant increase in only one of the aroma compounds analysed in this study, this component ethyl butanoate increased by 26% ($P<0.001$) relative to the Control wines, imparting a greater stone fruit (peach) aroma to the wine (Table 7b.4). ACE maceration resulted in significant differences in four of the 16 aroma compounds analysed relative to the Control wines which were spread across all three classes of aroma compounds, each one having a fruity descriptor: the ethyl ester, ethyl butanoate, increased by 21% ($P<0.001$); the higher alcohol, butanol, increased by 30% ($P=0.001$); the acetates also increased, 2-methylpropyl acetate by 14% ($P=0.22$) and 2&3-methylbutyl acetate by 27% ($P=0.17$). These compounds impart banana and floral aromas to the wine. For the ACE and Submerge treatment wines, only one aroma characteristic was significantly affected relative to ACE wines, that being a larger percentage (16%; $P=0.002$) of 2-methylpropanol which imparted more fusel aromas (Table 7b.4).

Table 7b.4 Aroma compound ₸response ratio in Pinot Noir wine from four maceration treatments: Control; Submerge Cap; ACE and ACE and Submerge, at six months bottle age (230 days post-inoculation).

	Control	Submerge	ACE	ACE & Submerge	*P-value*	Aroma Descriptor
Ethyl Esters						
ethyl acetate	68.6 b	55.4 a	69.7 b	55.9 a	*<0.001*	sweet, tart, volatile acid, nail polish
ethyl propanoate	0.90 b	0.66 a	0.86 b	0.63 a	*<0.001*	fruity
ethyl 2-methylpropanoate	7.90 b	6.24 a	5.69 a	7.06 ab	*0.006*	fruity, sweet, apple
ethyl butanoate	2.12 a	2.64 b	2.57 b	2.69 b	*<0.001*	fruity, peach
ethyl 2-methylbutanoate	0.44 b	0.35 a	0.29 a	0.34 a	*<0.001*	sweet, fruit, honey
ethyl 3-methylbutanoate	0.06 b	0.05 b	0.04 a	0.05 b	*<0.001*	berry, fruity
ethyl hexanoate	0.28	0.28	0.26	0.27	*0.779*	green apple, fruity, wine
ethyl octanoate	2.63	3.20	2.61	2.95	*0.077*	red cherry, raspberry, cooked fruit
ethyl decanoate	0.17 a	0.21 b	0.20 ab	0.18 ab	*0.029*	fruity, black cherry, chocolate

Acetates						
2-methylpropyl acetate	1.22 ab	1.06 a	1.39 b	1.23 ab	*0.022*	banana, fruity, floral
2&3-methylbutyl acetate	10.1 a	13.0 b	12.8 b	12.3 ab	*0.017*	banana, fruity
hexyl acetate	0.06	0.06	0.06	0.06	*0.375*	sweet, perfume, floral
Alcohols						
2-methylpropanol	16.9 a	16.7 a	17.0 a	19.7 b	0.002	fusel, spirituous, nail polish
butanol	0.10 ab	0.10 a	0.13 c	0.11 bc	0.001	fruity, fusel, spirituous
2&3-methylbutanol	150	159	162	162	0.115	nail polish
hexanol	4.86 b	3.47 a	5.01 b	3.71 a	<0.001	grape juice, green grass

Ŧ Ratio of the compound peak area to the related internal standard peak area. Data are mean values (n=4). Results in the same row with different letters are significantly different according to independent samples T-Test (P<0.05).

7b.4.4 Sensory evaluation of wine

The treatment mean scores for appearance, aroma and palate attributes showed a number of clear differences between treatments. Mean scores for attributes with significant differences between treatments are shown in Figure 7b.1 for appearance and aroma and in Figure 7b.2 for palate. The appearance attribute *Red* was rated significantly higher in the two ACE treatments than in the Submerge Cap treatment, which was higher than the Control wines (Figure 7b.1). ACE treated wines with were also scored higher for *Dark fruit* aroma and palate attributes, than were Submerge Cap or Control wines. Control wines were scored significantly higher for *Red fruit* aroma than the other treatments, while for the *Red fruit* palate attribute, the Control and Submerge Cap treatments were significantly higher than the ACE treatments. Control wines were also scored highest for *Confection* and lowest for *Drain* aroma. The Submerge Cap treatment was scored highest for *Drain* and *Sweaty/cheesy* aroma attributes (Figure 7b.1).

ACE and Submerge Cap wines were scored significantly higher for *Stalky* (palate) than either Submerge Cap or Control wines, while ACE wines were scored significantly higher for *Stalky* (palate) than the Control wines (Figure 7b.2).

The ACE treated wines, whether Submerged or plunged twice daily, scored significantly higher for *Astringency* than 'Submerge Cap' wines, which in turn were higher than Control wines. ACE wines were also scored significantly higher than Submerge Cap wines for *Bitter* (palate), which was higher than the Control wines. The ACE and Submerge treatment wines were not significantly different to either ACE or Submerge Cap treatments, but were significantly higher than the Control wines (Figure 7b.2).

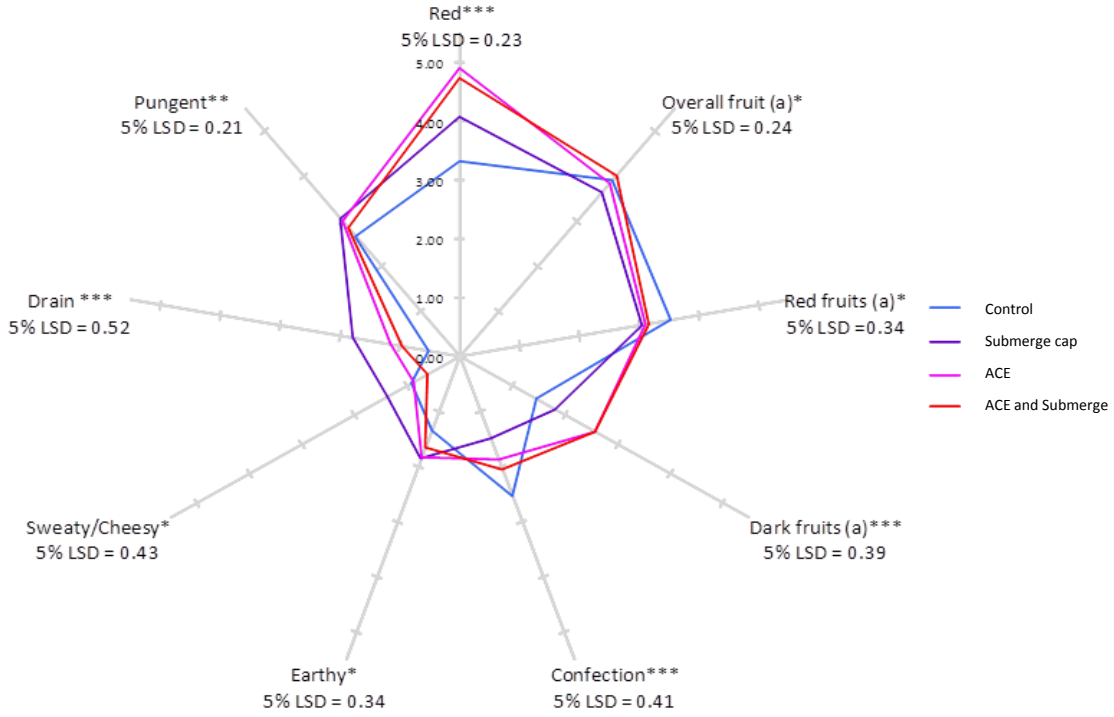

Figure 7b.1. Mean scores (n=4) for aroma attributes of Pinot Noir wine from four maceration treatments at six months bottle age (230 days post-harvest). Treatments: Control; Submerge Cap; ACE; ACE and Submerge *p < 0.05; **p < 0.01; ***p < 0.001; 5% LSD determined using Fisher's pot-hoc test for Least Significant Difference (LSD).

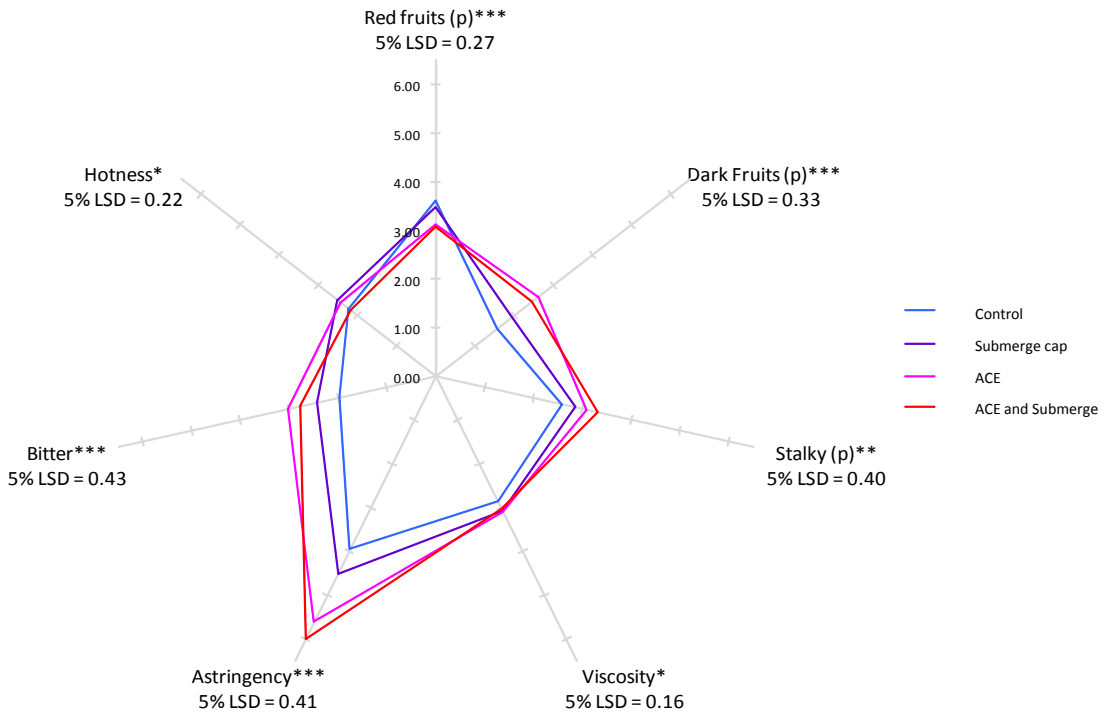

Figure 7b.2 Mean scores (n=4) for palate attributes of in Pinot Noir wine from four maceration treatments at six months bottle age (230 days post-harvest). Treatments: Control; Submerge Cap; ACE; ACE and Submerge;*p < 0.05; **p < 0.01; ***p < 0.001; 5% LSD determined using Fisher's pot-hoc test for Least Significant Difference (LSD).

All of the cap management treatments imposed in this study influenced the sensory properties of the Pinot Noir wines when compared to the Control wines. Control wines were scored higher for *Red fruits* (aroma and palate) and *Confection* scores, as well as lower *Red* (appearance), *Dark fruits* (aroma and palate) and *Stalky, Viscosity, Astringency*, and *Bitter* palate characteristics compared to other treatments. ACE treated wines were

highest in *Red* colour (appearance), *Dark Fruits, Stalkiness, Viscosity, Astringency* and *Bitter* characters, while for many attributes the Submerge treatment wines were between the Control the ACE treated wines. Using the significant attributes for PCA, a clear picture of the treatments emerged (Figure 7b.3).

Separation along the PC1 axis showed clear differentiation of Control wines from all other treatments. Along this axis, separation was influenced by *Red Fruits* (aroma and palate) and *Confection* in the negative direction and by *Red* (colour), *Dark fruits* (aroma and Palate), *Earthy* aroma, *Stalky*, *Viscosity*, *Bitter* and *Astringency* palate attributes in the positive direction. As noted from Figure 7b.2, this reflects higher scores for the Control for *Red fruits* and *Confection* and lower scores for *Red*, *Dark fruits*, *Stalky*, *Viscosity*, *Astringency* and *Bitter*.

Separation along PC2 was influenced by *Overall Fruit* and *Confection* attributes in the negative direction and by *Sweaty/cheesy*, *Drain*, *Pungent* (aroma) and *Hotness* attributes in the positive direction. This PC separated the Submerge treatment from other treatments and in particular replicate 1 of this treatment which was scored higher for *Sweaty/cheesy* and *Drain* in particular and lower for *Confection*. The ACE and ACE and Submerge treatments were also separated from one another – ACE wines had slightly higher PC1 and PC2 scores than ACE and Submerge wines. Among the ACE wines, replicates 1 and 2 had higher PC2 scores and replicates 3 and 4 had higher PC1 scores. Although PC3 was statistically important in the analysis of this data it does not clarify the separation between treatments, as can be seen from Figure 7b.3 (b). This may be due to some differences across all replicates for some attributes such as *Red* (appearance), Dark fruit (aroma) and Confection, while for the attributes *Sweaty*/*cheesy* and *Drain* there was a single replicate of the Submerge treatment which was rated higher than other replicates.

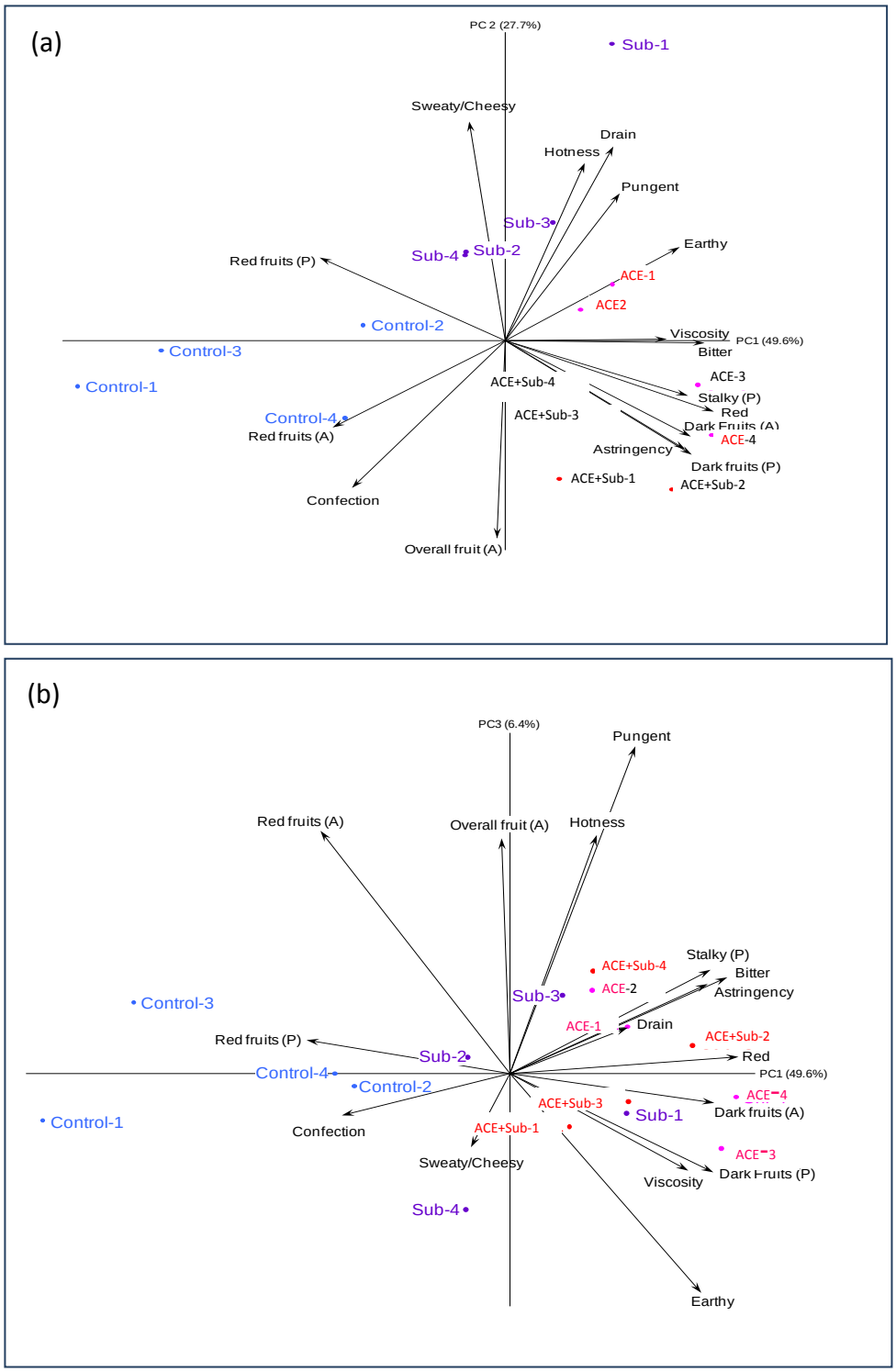

Figure 7b.3: Scores and loading bi-plot for significant sensory attributes scored for Pinot Noir wines made with different cap management treatments. (a) PC1 and PC2 account for 74% of the variance in the model; (b) PC1 and PC3, add a further 6.4% of variance in the model. Total across the three PCs is 84% of explained variance. Treatments: ACE, Accentuated Cut Edges; Sub, Submerge Cap; ACE+ Sub, Accentuated Cut Edges and Submerged Cap; numbers after treatment name define the replicate.

All of the cap management treatments influenced the sensory properties of the Pinot Noir treatment wines compared to the Control wines. While there was some variability between replicates, there were clear differences between treatments. Control wines were scored higher for *Red fruits* (aroma and palate) and *Confection* scores as well as lower *Red* (colour), *Dark fruits* (aroma and palate) *Stalky* (palate), *Viscosity*, *Astringency*, *Bitter* compared to other treatments. The ACE and the ACE and Submerge treatments were highest in the *Red* (appearance), *Dark Fruits*, *Stalky*, *Viscosity*, *Astringency* and *Bitter*, while the scores for many attributes of the Submerge treatment lay between the Control and the ACE and Submerge and ACE treatments. In the Submerge treatment, one replicate (Replicate 1) was scored higher than all other wines for *Sweaty*/*Cheesy* and *Drain* attributes, these characters possibly had a masking effect on other attributes.

7b.4.5 Comparison of sensory and chemical analyses

Linear regression analyses were conducted to assess the relationship between the sensory attributes of Pinot Noir wine as scored by the panel of 12 judges and phenolic parameters measured by chemical analyses (Table 7b.5).

There were strong positive correlations between red appearance of the wine and both colour density and concentration of non-bleachable pigment (r^2=0.95; P<0.001) measured by UV-VIS spectroscopy (Table 7b.5). While there was no correlation between red appearance and wine hue, there was a significant negative correlation between red appearance and hue SO_2 (r^2=0.43; P=0.005) (Table 7b.5). Colour density was also positively correlated with dark fruit aroma (r^2=0.72; P<0.001)and flavour (r^2=0.85; P<0.001) . Non-bleachable pigment was correlated with both red and dark fruit flavours, astringency and bitterness, while tannin was strongly correlated with astringency and bitterness (Table 7b.5).

The results show that the non-bleachable pigment component of the wine which represented both pigment-tannin adducts and pyranoanthocyanins, was strongly correlated with significant sensory properties of the wine.

Table 7b.5. The relationship between sensory attribute score and phenolic parameters measured spectroscopy of Pinot Noir wines at 6 months bottle age.

Sensory attribute	Phenolic parameter	r^2	P-value
red appearance	colour density	0.95	<0.001
red appearance	non-bleachable pigment	0.95	<0.001
red appearance	% NBP[Ŧ]	0.57	0.001
red appearance	hue	0.09	0.16
red appearance	hue SO2	0.43*	0.005
red fruit (aroma)	colour density	0.66*	0.02
red fruit (palate)	colour density	0.43*	0.002
red fruit (palate)	non-bleachable pigment	0.67	<0.001
dark fruit (palate)	colour density	0.85	<0.001
dark fruit (palate)	non-bleachable pigment	0.85	<0.001
dark fruit (aroma)	colour density	0.72	<0.001
astringency	non-bleachable pigment	0.87	<0.001
astringency	tannin	0.97	<0.001
bitterness	non-bleachable pigment	0.66	0.001
bitterness	tannin	0.66	<0.001

Data are linear regression values (r^2) for pairwise comparisons (P≤0.05). *Indicates a negative correlation. [Ŧ]NBP (non-bleachable pigment)

7b.5 DISCUSSION

Pinot Noir wines are lightly pigmented red wines whose reputation for bottle ageing can be poor. Following earlier work which demonstrated the significant impact of reducing the grape skin particle size using ACE maceration on the phenolic components of Pinot Noir wines associated with colour stability (Chapter 7a), this investigation examined both the phenolic and sensory responses of ACE wines and those in which the pomace cap was submerged during fermentation. The cap management techniques described in this study resulted in significant changes not only in the phenolic composition of the wine but in the appearance, aroma and taste of the wine. Colour density increased by more than 50% with the excess anthocyanin released by applying ACE maceration on day 1 reflecting an increase in non-bleachable pigment in ACE wines as the ferment progressed; higher levels of fruity components, notably banana and peach, were determined by aroma analysis; while sensory evaluation pointed to greater intensity of red colouration and dark fruit flavours. Along with more intense colour, flavour and aroma properties, the maceration techniques also imparted distinctly astringent and bitter characters to the wine, which are most likely to be attributed to the tannin concentration on which there was an immediate impact that was maintained through to six months bottle age.

Submerged Cap: A major advantage of using Submerged Cap management is a labour saving one; in experimental trials that use small ferment sizes this technique has also been shown to give remarkably replicable results which in turn highlight treatment effects (Dambergs and Sparrow, 2011). In this study, wines made by submerging the cap throughout fermentation were more intensely coloured than Control wines made with twice daily plunging of the cap as noted by the sensory panel, and the majority of this colour was attributed to there being a 25% increase in the concentration of stable

pigments. Aroma properties associated with Submerged Cap wines showed a higher proportion of fruity aromas including peach, dark cherry and banana, however these fruity aromas were offset by fusel and sweaty/cheesy aromas indicative of a higher proportion of 2-methylpropanol, butanoic, hexanoic and octanoic acids respectively, which were also noted by the sensory panel. Overall, Submerged Cap wines resulted in an improvement in the fruitiness, colour intensity and colour stability of Pinot Noir wines while the unpleasant aromas detected may have been mitigated by occasional stirring of the fermenting must, with the introduction of more oxygen promoting the formation of ethyl esters (Fang and Qian, 2005).

ACE maceration: The impact of reducing skin particle size was to significantly increase the colour density and the proportion of blue-purple pigmentation in the wine. Non-bleachable pigment concentration was almost double that of the Control wines, suggesting that changes in wine hue with bottle age would be modest. ACE wines had fruity characters similar to the Control wines, which were attributed to ethyl esters, yet showed more banana and floral fruit aromas, due to an increase in three of the four acetates analysed. The sensory panel found that either submerging the pomace cap or cutting it into smaller fragments by ACE maceration, increased both the astringency and the bitter flavours in the wine.

ACE and Submerge Cap: Cutting the pomace cap by ACE maceration was apparently the most significant treatment effect, as combining ACE maceration and Submergence of the cap caused little difference in either the wine phenolic composition or the sensory properties measured.

The relative extractability of the grape tannins and the maceration treatment (or in this case, the cap management technique) determines the proportion of skin or seed derived

tannins that occur in the wine matrix, (Vidal et al., 2003a, Cortell et al., 2005, Koyama et al., 2007a) and optimising these proportions is essential for wine quality (Bautista-Ortin et al., 2007). As reported by Peyrot des Gachons and Kennedy (2003), the rate of seed tannin extraction appears to accelerate during the latter half of Pinot Noir vinification whereas the concentration of skin tannin is likely to have a strong influence on final wine tannin.

In a number of studies, the phenolic composition of seeds was found to be responsible for both astringency and bitterness in the wines (McRae and Kennedy, 2011, Vidal et al., 2003a, Canuti et al., 2012). However, astringency in red wines has generally been associated with large tannin polymers which are usually derived from the skin (Fischer and Noble, 1994, Vidal et al., 2003a, Vivas et al., 2004, Adams, 2006, Kennedy, 2008, Bindon et al., 2010b, Neves et al., 2010, McRae et al., 2013). It is noteworthy that the wines analysed during the course of this study had not undergone a malo-lactic fermentation, the lack of which may have exacerbated the sensation of astringency in the wines. Bitter flavours in wine have been associated with tannin monomers and small oligomers (Noble, 1998, McRae et al., 2013). Investigating red grape varieties (Mattivi et al., 2009) found that Pinot Noir grapes were three to four times higher in proanthocyanidin monomers than other red grape varieties suggesting that there may be a much higher proportion of bitter flavoured proanthocyanidin monomers in Pinot Noir wines. As noted in experimental results described in chapter 4 and a recent report by Bindon (2013b), tannin extraction from grape tissues is promoted in proportion to the anthocyanin concentration of the wine matrix which subsequently results in the formation of a higher concentration of non-bleachable pigment in the wine as described in this study. It is also possible that ACE maceration caused sufficient disruption to the skin and pulp tissues so that the usual binding of tannin molecules by polysaccharide structures of cell wall fibres (Bindon et al., 2010a, Bindon et

al., 2010b) was reduced to such an extent that a significant portion of the extracted tannins remained solvent in the wine matrix and were not discarded with the grape marc when the wine was pressed.

7b.6 CONCLUSION

This study showed that both reducing the particle size of the floating pomace cap and submerging the pomace cap of Pinot Noir in the initial stages of fermentation had considerable impact on both the phenolic and sensory properties of the wine. At six months bottle age ACE wines were more intensely coloured, had greater fruit intensity, and had a greater concentration of stable colour pigments than wines made by the conventional cap management technique of plunging the cap twice daily. The increase in the perimeter of skin edges probably accounted for the more rapid release of anthocyanins and skin tannins from ruptured cells in the early part of vinification and in turn these molecules polymerised to form non-bleachable pigments. The increase in the sensory properties of both astringency and bitterness may be attributed to the higher extraction rate of tannins from both skin and seed grape tissues. ACE maceration treatment was successful in turning a wine that was in the lowest end of the Pinot Noir tannin range to one at the highest end of the range where the Burgundies lie. The early development of wine polyphenols also gives oenologists the option of early press off from the pomace to complete fermentation without further exposure to the seeds this step may include barrel fermentation.

7b.7 SUBSEQUENT RESEARCH FOCUS

The benefits of improved colour stability as a consequence of reducing the skin particle size of Pinot Noir grapes are clear. *Dark fruit* characters were also more pronounced in ACE wines. Although ACE macerated wines were found to have increased levels of *Bitter* and *Astringency* characters, these are most likely to have been contributed by seed derivatives due to the advanced extraction of seed tannins facilitated by their physical release from the pulp tissues. Experiments described in this Chapter demonstrated that the majority of colour pigments and skin tannins leak from the broken edges of the skin fragments immediately following the cutting procedure. Consequently, the exploration of early press off from the pomace to counteract the more ready release of seed tannin to the wine matrix is an option worthy of further investigation.

In addition, individual phenolic parameters analysed by spectroscopy were found to be closely correlated with particular flavour attributes. This finding has not been reported previously and makes the use of simple, chemical analyses interpreted by spectroscopy and chemometrics, a valuable tool for both the research scientist and the winemaker. The recognition of specific wavelengths at which absorbance decreases as seeds are removed (Chapter 6b), coupled with the phenolic and sensory attributes for the cap management treatments described in Chapter 7, provide a further opportunity for research.

8

PHENOLIC QUALITY INDEX DEVELOPED TO DIFFERENTIATE SEED AND SKIN PHENOLICS IN PINOT NOIR WINE

8.1 ABSTRACT

The unique phenolic profile of Pinot Noir wines makes this variety one of the most challenging to vinify. Meta-analysis of winemaking trials conducted over three consecutive years using fruit from *Vitis vinifera* cv. Pinot Noir revealed the relative contributions to the phenolic composition of Pinot Noir table wine from grape skin and seed tissues. Spectral interpretation at six wavelengths 270, 280, 320, 520 and 580 nm that have been reported in the literature to represent signature classes of phenolic acids, flavan-3-ols and colour pigments was used to define an index of phenolic quality (PQI). Using an algorithm, the PQI for wines was found to reflect the maceration technique employed. In wines aged for six months in the bottle PQI increased by 38% relative to control wines when vinification involved thermo-vinification by microwave or extended maceration, whereas freeze-thaw maceration reduced PQI by 28%. The addition of commercial grape seed tannin reduced PQI of the wines by 20% relative to control wines. The PQI has the potential to be used as an aid to decision making during vinification, with the aim of optimising wine quality in response to fruit attributes and the preferred wine style.

8.2 INTRODUCTION

Wine tannins and anthocyanins contribute significantly to properties of red wine such as astringency, mouth-feel and colour (Etaio et al., 2009, Herderich and Smith, 2005, Vidal et al., 2004a, Vidal et al., 2004b, McRae et al., 2012, McRae et al., 2010, McRae and Kennedy, 2011, McRae et al., 2013). Tannins are derived from both the skin and seed of wine grapes, while anthocyanins are derived from grape skins. Pinot Noir grapes have thin skins and therefore inherently low concentrations of anthocyanins (Girard et al., 1997, Sacchi et al., 2005, Mattivi et al., 2009, Neves et al., 2010) so that colour development and stability in

the wine are often compromised relative to wine made from other red grape varieties (Boulton, 2001, Lesica and Kosmerl, 2006, Skogerson et al., 2007, Versari et al., 2007, Versari et al., 2008, Sparrow et al., 2013a, Sparrow et al., 2013c). Pinot Noir grapes contain no acylated or coumarylated anthocyanins, with the result that in addition to having low concentrations of anthocyanins, the anthocyanins are less stable (Mazza et al., 1999, He et al., 2012b). During winemaking, tannins bind with anthocyanins to form stable colour pigments which also contribute to the flavour attributes that characterise the wine style. Grape skin tannins are more valuable in the development of wine sensory properties such as astringency than are seed tannins (McRae et al., 2010). However as a result of their thin skins, the ratio of skin tannins to seed tannins in Pinot Noir grapes is low (Sacchi et al., 2005, Mattivi et al., 2009, Neves et al., 2010). Consequently, Pinot Noir wine quality is strongly correlated to the skin tannin and anthocyanin content of grapes at harvest and to the proportion of tannin extracted from the two major grape tissue sources during winemaking (Cortell and Kennedy, 2006).

Throughout this investigation samples collected from wines made using a range of maceration manipulations were analysed and interpreted to estimate wine colour (Mercurio et al., 2007) and tannin (Dambergs et al., 2012b). The objective of this study was to use the data to examine the potential for development of a simple procedure by which phenolic compounds derived from the skin or seed of the grapes could be differentiated.

8.3 MATERIALS AND METHODS

8.3.1 Grape composition

Five different clones of *Vitis vinifera* cv. Pinot Noir were used in 15 individual experiments over three years. A 200 g sample of berries from each clone was analysed for grape colour (Iland et al., 2004) and total tannin content (Dambergs et al., 2012b).

Grape skin and seed tissues were also isolated from three replicate samples of fresh grapes and the phenolic profile of each tissue type identified. Samples of 50 g of each grape tissue were homogenised in 100 mL of sucrose buffer (24% sucrose, 8 g/L tartaric acid adjusted to pH 3.3 with 5M sodium hydroxide) at 8000 rpm for 20 seconds in a Retsch Grindomix GM200 homogeniser with an S25 N-18G dispersing element (Janke & Kunkel GmbH & Co, Germany) fixed with a floating lid. After being incubated in the dark for one hour, absorbance was read across the ultra-violet (UV) spectrum from 200 to 350 nm. A second set of samples were prepared for determination of total tannin using the methyl cellulose precipitation (MCP) assay described by Sarneckis et al. (2006). The methyl cellulose precipitates were resuspended in 10 ml of 1.0 M HCl and the absorbance of these samples was read across the ultra-violet (UV) spectrum from 200 to 350 nm. Commercial preparations of grape seed tannin and grape skin tannin were also analysed using the MCP assay.

8.3.2 Microvinification protocol

Absorbance values used throughout this study were wines at 6 months bottle age that were made using submerged cap micro-fermentation techniques (Dambergs and Sparrow, 2011, Smart et al., 2012) as described in previous chapters.

In order to qualify the stable (non-bleachable) pigment content of the fermenting and finished wines, samples were diluted 1:10 in model wine solution (saturated solution of

potassium hydrogen tartrate in 12% v/v ethanol) containing 0.375% sodium metabisulphite and absorbance read at 520 nm and 580 nm.

8.3.3 Statistical analyses

Statistical analyses were conducted using GenStat 64-bit Release 16.1 Copyright 2013, VSN International Ltd. Means for each of the wavelength ratios and the Phenolic Quality Index, was calculated using one-way ANOVA was followed by post-hoc analysis using Fisher's Protected Least Significant Difference (LSD) test ($P \leq 0.05$).

8.4 RESULTS AND DISCUSSION

8.4.1 Grape composition

For each of the five clones of *Vitis vinifera* cv. Pinot Noir examined in this study, the average ratio of tannin to anthocyanin was 12:1, with the exception of the seedless clone, in which the ratio was 1.5:1 (Table 8.1). This is in sharp contrast to the ratio of tannin to anthocyanin in Cabernet Sauvignon and Shiraz grapes which has been reported to be 2.1:1 (Holt et al., 2008) and 1.6:1 (Ristic et al., 2010) respectively. In order to make a high quality Pinot Noir wine, balancing the phenolic composition of the wine is vital. Not only do polyphenols contribute to a wide range of wine sensory properties, they also determine the ageing potential of the wine. Maceration techniques and the addition of oenotannins employed during winemaking effectively produce wines that range in style from those that are light textured with red fruit characters, to more intensely coloured wines with dark fruit characters.

Table 0.5 Phenolic composition of five clones of Pinot Noir grapes over a 3-year period

Data are mean and standard deviation (STDEV) (n = 4) for grapes from each *Vitis vinfera* cv. Pinot Noir clone' in each year.

Thesis Chapter	Pinot Noir Clone	Vintage	Anthocyanin (mg/g ŦFW)		Tannin (mg/g ŦFW)		ƀTan: Antho ratio
			Mean	STDEV	Mean	STDEV	
2 and 7b	D5V12	2013	0.58	0.03	6.87	0.2	11.8
4	G5V15	2011	0.40	0.01	4.88	0.2	12.2
5	115	2012	0.63	0.1	7.03	0.4	11.2
5	115	2012	0.69	0.1	8.68	1.2	12.6
5	D5V12/D4V2	2012	0.61	0.2	7.82	1.9	12.8
6a	seeded	2011	0.63	0.1	5.87	0.5	9.3
6a and 6b	G5V15	2012	0.68	0.01	6.84	0.3	10.1
7a	G5V15	2011	0.38	0.04	4.68	0.7	12.3
7a	115	2012	0.63	0.1	7.76	0.4	12.3
Average							11.6±1.2
6a	seedless	2011	1.98	0.1	2.99	0.2	1.5

ŦFW, fresh weight; ƀTan:Antho ratio, ratio of tannin concentration to anthocyanin concentration in grapes

8.4.2 Spectral properties of phenolic standards

Spectra of standard solutions (0.4 mg/L) of gallic acid, caffeic acid, catechin, epicatechin and epicatechin-gallate were prepared in 1.0 M HCL (Figure 8.1A). As each of these phenolic components had a significant absorbance peak at 220 nm, with gallic acid being 55% greater than the other compounds, the spectra were normalised around this peak to emphasise the differences in absorbance at other wavelengths (Figure 8.2B). Notable differences included the broad absorbance range for caffeic acid from 280 to 334 nm with peak at 320 nm and the peak at 280 nm for catechin, epicatechin and epicatechin-gallate.

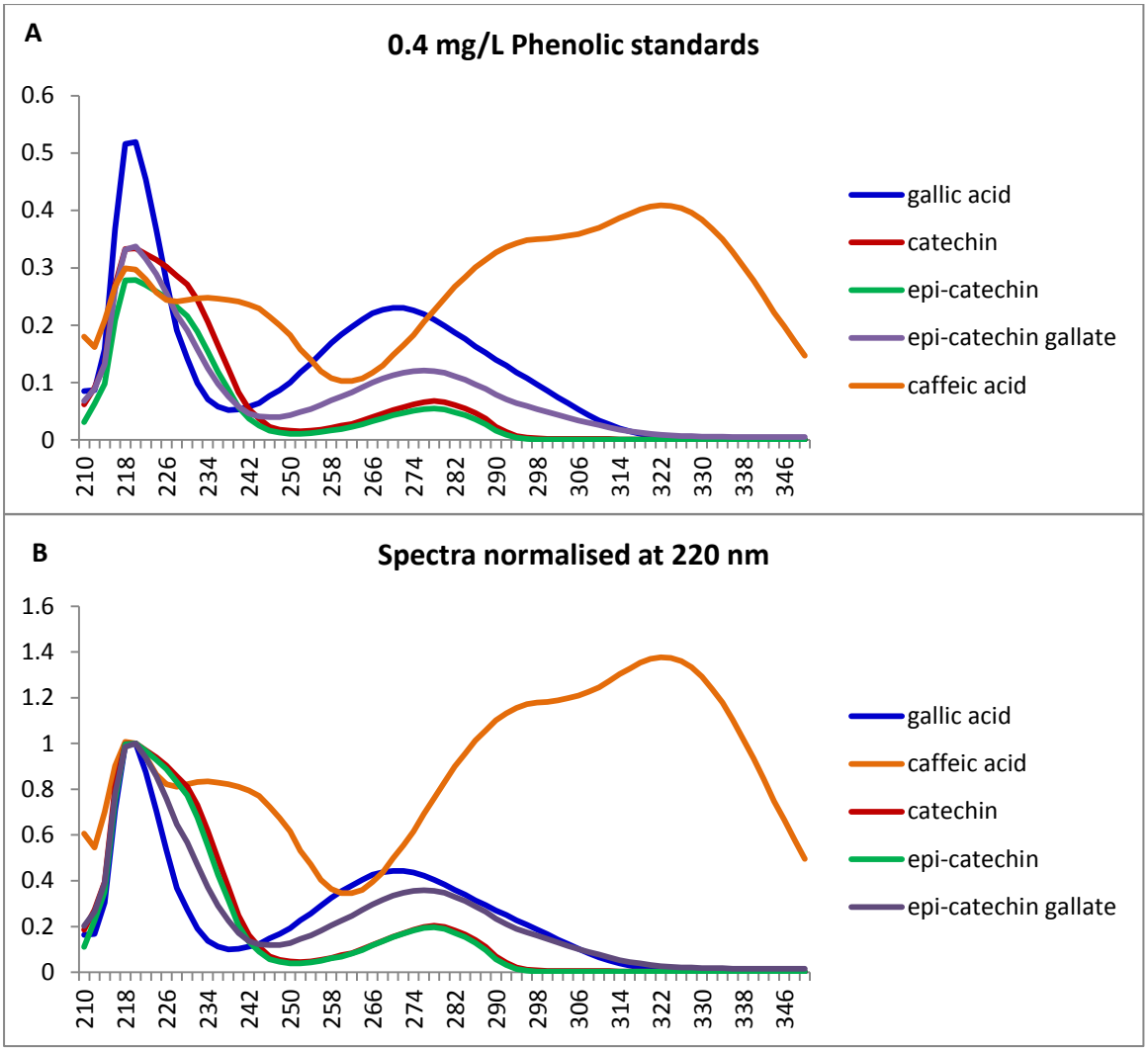

Figure 0.9 Absorbance spectra of phenolic standards from 210 to 350 nm

A. Each component was dissolved in 50% acidified ethanol and diluted in 1.0 M HCl to concentration (0.4 mg/L). B. Absorbance spectra for each component at 0.4 mg/L normalised at absorbance 220 nm.

Isolated skin and seed tannins extracts were fractionated using MCP analysis and showed significant peaks at 220 nm, which Chanwitheesuk et al. (2005) identified as an absorbance peak for gallic acid which has been reported to occur in 3–fold higher concentration in grape seeds than in grape skins (Yilmaz and Toledo, 2004). However absorbance at 220 nm has also been associated with proanthocyanidin subunits (Figure 8.1A) which complicates the identification of the source of phenolics at this wavelength. Absorbance at 320 nm

detected in grape tissues has been identified as caffeic acid which is a derivative of caftaric acid and occurs in grape skin and juice but not in grape seeds (ETSLaboratories, 2011)

When the spectra were normalised at 280 nm, the peak the absorbance value at 320 nm was higher for skin tannin than for seed tannin extracted using MCP (Figure 8.2B).

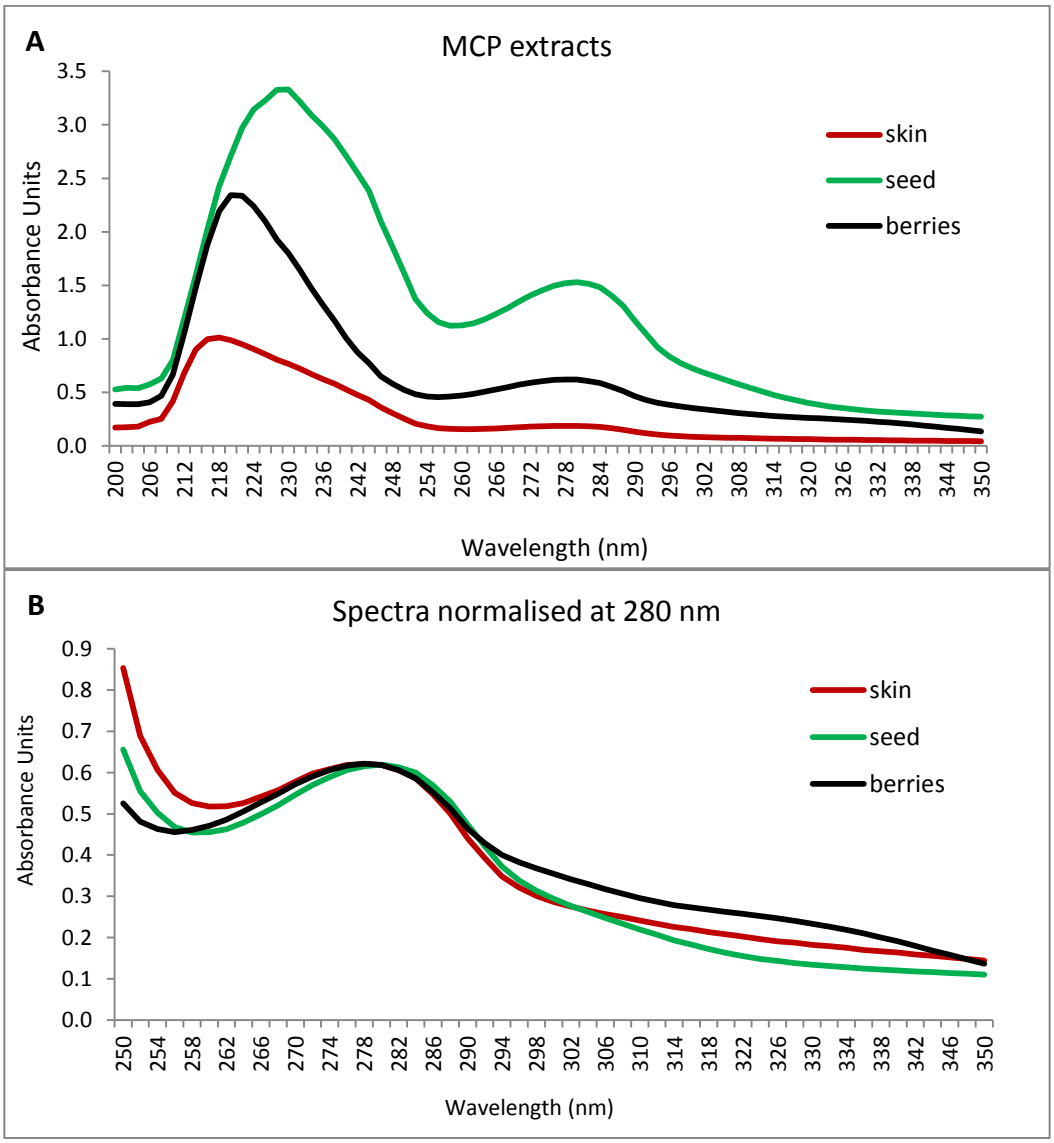

Figure 8.0 Absorbance spectra of skin and seed extract MCP tannin isolates.

A. Isolated skin and seed tissues were extracted in 50% ethanol and fractionated by MCP and compared to whole berry extracts; B. Absorbance spectra for each component normalised at absorbance 280 nm.

Pinot Noir wines made with a double complement of one grape tissue confirmed the higher absorbance at 320 nm when skins were doubled compared to seeds (Figure 8.3A). Of particular note when the spectra are normalised at 280 nm is that wines made with a double complement of pulp selectively remove seed tannin (Figure 8.2B) as described in Chapter 4.

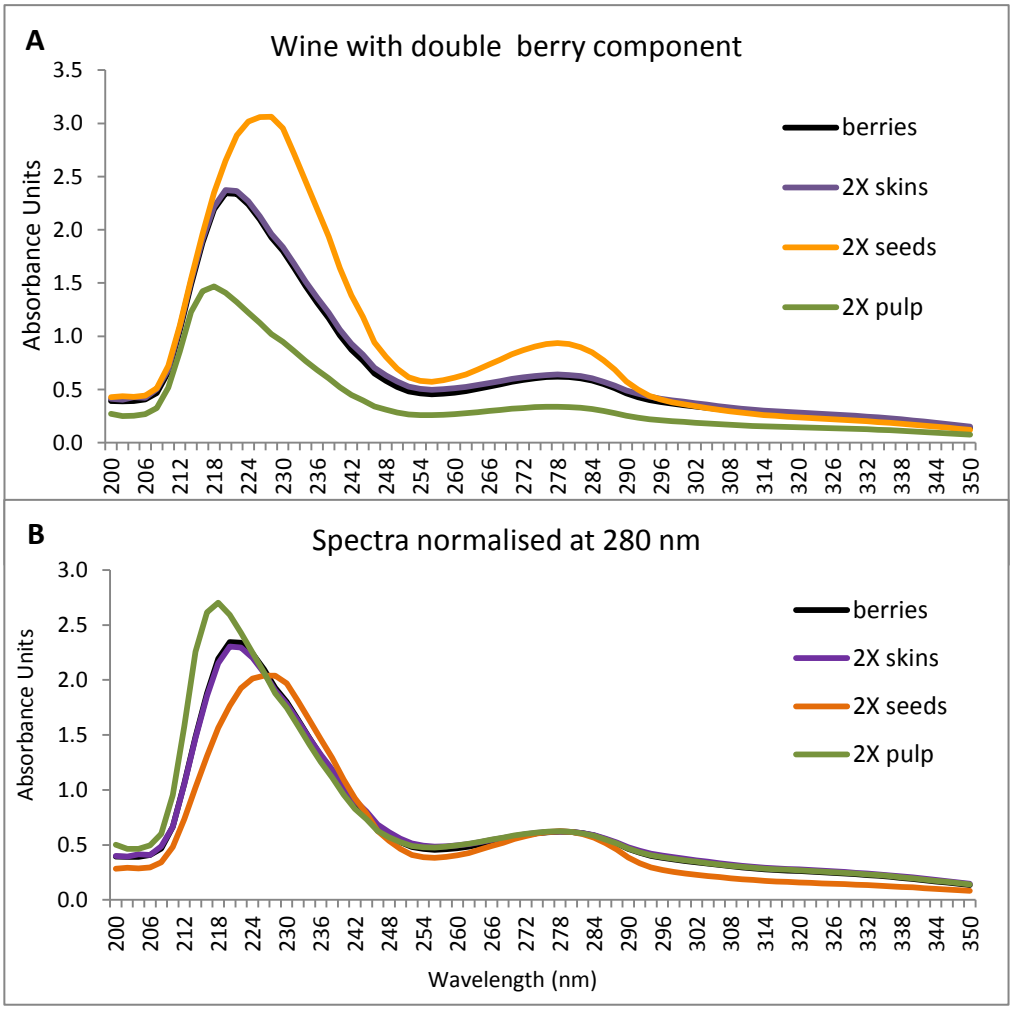

Figure 8.3 Absorbance spectra of wines with double berry component at six months bottle age, spectral range 250 to 350 nm.

A. Wines at 6 months bottle age whole berry ferments compared with wines made with a double complement of skins or seeds. B. Absorbance spectra for each wine normalised at absorbance 280 nm.

In addition to definitive absorbance in the lower ultraviolet range, absorbance at 270 nm corresponds to peak absorbance by epi-gallocatechin, a proanthocyanidin which occurs in grape skins but not in grape seeds (Kennedy et al., 2001, Vivas et al., 2004, Cortell and Kennedy, 2006, Koyama et al., 2007a, Lee et al., 2008, Mattivi et al., 2009, Wei et al., 2009, Bindon and Smith, 2013b, McRae et al., 2013). Conversely, absorbance at 280 nm corresponds with absorbance of catechins and epi-catechins (Donovan et al., 1999) which are present in all grape tissues, but are most highly concentrated in the grape seed (Prieur et al., 1994, Mattivi et al., 2009, Bindon and Smith, 2013b) and occur in much higher

Several phenolic components were found to have absorbance readings at similar wavelengths, consequently the ratio of absorbance at definitive wavelengths better differentiated skin derivatives from seed derivatives. The ratio of absorbance at 320 nm and 280 nm was indicative of the relative proportions of phenolic acids derived from skin and seed and the ratio of absorbance of 270 nm to 280 nm represented the flavan-3-ols, epigallocatechin (Wei et al., 2009) relative to catechin, epicatechin and epi-catechin gallate (Cortell and Kennedy, 2006, Wei et al., 2009).

Using the four selected wavelengths: 220, 320, 270 and 280 nm, the absorbance ratios A320:A220 and A270:A280 were calculated for grape tissue samples from tannins that had been fractionated using methyl cellulose precipitation (Table 8.2). Rapid tannin analysis was also used to estimate tannins isolated from skin and seed tissues (Dambergs et al., 2012b) to compare against the more complicated MCP analysis. The rapid tannin assay was conducted on commercial skin and seed tannin extracts. Using both methods the ratio of absorbance at 320:280 nm and the ratio of absorbance at 270:280 nm was significantly higher for skin extracts than for seed extracts.

Table 8.2. Absorbance values of isolated seed and skin extracts at selected wavelengths

Tannin source	ratio 320/280	ratio 270/280
Methyl Cellulose Precipitates		
*skin isolate	0.34	0.97
*seed isolate	0.26	0.87
P-value	*<0.001*	*<0.001*
¥commercial skin tannin	0.35	0.94
¥commercial seed tannin	0.25	0.88
P-value	*0.014*	*0.005*
Unfractionated Extracts		
¤skin isolate	0.45	0.98
¤seed isolate	0.12	0.87
P-value	*0.004*	*<0.001*

Isolated *skin and *seed tissues and commercial ¥skin and ¥seed tannins fractionated with methyl cellulose; unfractionated 50% ethanol extracts of isolated skin and ¤seed preparations diluted in 1 M HCl. Data are mean values (n=3); values for different tissue types for each preparation are significantly different at $P \leq 0.05$.

However the situation in wine is more complicated, as the extraction of tannins occurs under relatively passive conditions. Absorbance at 280 nm is compromised by the relative proportion of tannin that is extracted from each tissue type. When seed tannin is present in excess in the wine, the ratio of absorbance at 320/280 nm decreases as does the ratio of absorbance at 270/280 nm. The presence of excess skin tannins increases the ratio A320/A280 and A270/A280 by a small amount (Table 8.3). In order to qualify stable pigments in the wine a third ratio was introduced. Free anthocyanins have peak absorbance at 520 nm in model wine solution. A second absorbance peak at 420 nm is

indicative of yellow/orange pigments formed as the anthocyanin (malvin) reacts with flavan-3-ols (Somers and Evans, 1977, Liao et al., 1992, Mercurio et al., 2007, Ivanova et al., 2011). As wine ages, anthocyanins combine with other molecules in the wine to form stable wine pigments, which are not bleached when sulphur dioxide additions to the wine are made during vinification. One class of stable pigments, in which anthocyanins become polymerised with tannins are referred to as 'pigmented tannins' that are responsible for the red-brown hues in the wine (Somers and Evans, 1977, Boulton, 2001, Waterhouse, 2002, Cheynier et al., 2006). A second class of pigments are formed from reactions of anthocyanins with hydroxycinnamates such as caffeic acid to form pyroanthocyanins which contribute to blue-purple hues, some of which change to yellow-orange as the wine ages (Cheynier et al., 2006, Mateus et al., 2004, Morata et al., 2007). A higher proportion of a more stable (SO_2 resistant) class of pyranoanthocyanins may shift the wine hue from red-orange towards the blue-purple spectrum. The compounds have absorbance ranges over the wavelengths 510 to 800 nm with the flavanyl-vinyl-pyranoanthocyanins (portisins) in particular, being blue-purple in colour. The majority of pyranoanthocyanins in Pinot Noir have a peak absorbance at 580 nm (Alcalde-Eon et al., 2006, Chinnici et al., 2009, He et al., 2012b, Marquez et al., 2013, Mateus et al., 2004, Oliveira et al., 2009). The ratio of absorbance at 580 nm and 520 nm was selected to provide an estimate of stable red and blue-purple pigments (resistant to sulphur dioxide bleaching) in the wines. If preservation of the vibrant purple colour of stable pigments in young wines is seen as a positive trait, the ratio of 580/520 nm in the presence of sulphur dioxide will be a positive indicator.

Wines made in 15 experiments conducted during the course of this 3 year investigation were evaluated using the three definitive absorbance ratios. The wines were analysed at six months bottle age and are identified in Table 8.3 with corresponding Chapter

identification numbers. For the experiments described in Chapters 4, 5, 6a and 6b, wines were made from musts containing either: double complement of grape tissue fractions (Ch. 4); additional grape pomace or commercial skin tannin (Ch. 5); addition of fermented grape seeds or commercial grape seed tannins (Ch. 6a); or the removal of grape seeds (Ch. 6b). In each case, the ratio of absorbance 320/280 was higher, relative to the control wines (berries), in the presence of excess skins, and lower in the presence of excess seeds or seed tannins. The ratio of absorbance at 270/280 was higher, or not significantly different from the control wines (berries) in the presence of skins. Wines assayed in a model wine solution containing sodium metabisulphite confirmed the presence of pigments resistant to sulphur dioxide bleaching (non-bleachable pigments). Treatments that had been subject to microwave thermo-vinification, extended skin contact or skin cutting maceration techniques had a higher absorbance at 580/520 than control wines. It is noteworthy that for almost all of the maceration techniques conducted throughout this thesis the ratio of absorbance at 580/520 nm was less than 0.5, indicating that the red colour pigments dominated the colour of the wine more so than the blue-purple pigments.

The clear differences in the three absorbance ratios according to the maceration technique or source of additional tannin introduced during winemaking, allowed an index of phenolic quality (PQI) to be determined using an algorithm in a similar way to that described by Ristic et al. (2010) who calculated a score for potential wine quality based on grape analysis. These researchers differentiated skin derived proanthocyanidin subunits and seed derived proanthocyanidin subunits using phloroglucinolysis, and multiplied the ratio between the two different types of subunit by the anthocyanin concentration.

In the case of PQI, the three ratios of absorbance from the selected wavelengths were multiplied, and the total multiplied by 10 to magnify the sensitivity of the estimate:

$$\text{Phenolic Quality Index (PQI)} = (A320/280)*(A270/A280)*(580/520)*10$$

An advantage of using the PQI is that the chemical analyses required to calculate PQI are far less expensive and time consuming than those involved in phloroglucinolysis assays.

Table 8.3 shows the three absorbance ratios calculated from the six selected wavelengths and the PQI for 20 different maceration techniques undertaken during the investigations in this thesis.

Table 8.3. Must composition and selected absorbance ratios in Pinot Noir wine at 6 months bottle age.

Chapter	Winemaking trials	ratio 320:280	ratio 270:280	ratio 580:520	PQI
3	Control	0.59 a	0.92	0.39 c	2.11 b
	enzyme	0.58 a	0.91	0.37 c	1.95 b
	extended maceration	0.59 a	0.92	0.47 b	2.56 a
	microwave	0.51 c	0.92	0.69 a	3.25 a
	cold soak	0.56 b	0.92	0.39 c	2.00 b
	freeze-thaw	0.46 d	0.91	0.39 c	1.64 c
	P-value	*<0.001*	*ns*	*<0.001*	*<0.001*
4	Control	0.43 a	0.92 b	0.40	1.61 a
	2 x skins	0.43 a	0.94 a	0.39	1.60 a
	2 x seeds	0.42 b	0.90 c	0.42	0.98 b
	P-value	*<0.001*	*<0.001*	*ns*	*<0.001*
5	Control	0.50c	0.91 b	0.45	2.07
	Berries + LGST (20ml/L)	0.49 a	0.92 a	0.47	2.05
	Berries and 20% PN pomace	0.50 a	0.91 b	0.45	2.01
	Berries and 20% PN marc	0.45 b	0.91 b	0.45	1.89
	P-value	*<0.001*	*0.001*	*ns*	*ns*
5	Control	0.50	0.92	0.38 a	1.73 a
	PN + fresh PN Skins	0.50	0.92	0.37 a	1.67 a
	PN + fermented skins	0.47	0.91	0.35 b	1.52 b
	P-value	*ns*	*ns*	*0.012*	*0.022*

Chapter	Winemaking trials	ratio 320:280	ratio 270:280	ratio 580:520	PQI
6a	Control	0.53 a	0.93 a	0.37	1.92 a
	Berries + GST (4g/L)	0.50 b	0.92 b	0.38	1.67 b
	Berries + FPNS	0.45 c	0.90 c	0.39	1.52 c
	P-value	*0.007*	*0.006*	ns	*0.006*
6b	Berries seeded clone	0.49	0.90	0.40	1.79
	Berries seedless clone	0.44	0.93	0.49	1.99
	P-value	*<0.001*	*<0.001*	*0.001*	*0.022*
7a	Control	0.84	0.96	0.39	3.00
	Cut berries (ACE)	1.58	0.90	0.46	6.64
	P-value	*<0.001*	*<0.001*	*0.003*	*<0.001*
7b	Berries	0.84	0.96	0.20	3.00
	Berries with 15% seed removed	0.81	0.95	0.17	2.57
	P-value	*ns*	*0.006*	*ns*	*ns*
7b	Control	0.43 b	0.94 a	0.37 c	1.50 b
	Cut berries (ACE)	0.49 a	0.90 b	0.45 a	1.88 a
	Submerged berries	0.27 c	0.91 b	0.40 b	1.06 c
	P-value	*<0.001*	*0.002*	*<0.001*	*<0.001*

Wine samples were diluted 1:50 in 1.0 M HCl. Berries =Control; PN=Pinot Noir; LGST= liquid grape skin tannin; GST=grape seed tannin; FPNS= fermented Pinot Noir seed; PQI = Phenolic Quality Index. Data are mean values (n=4). For each values from each chapter in the same column that have different letters are significantly different at P≤0 .05).

The selected wavelengths gave an indication of the relative amounts of skin and seed derived phenolic compounds in wine subject to different maceration treatments and were used to calculate the PQI. The results from the experiment described in Chapter 3 in which six maceration treatments were compared, showed that while the maceration treatment apparently had no significant effect on the tannin subunit composition of the wine

described by the ratio 270/280, microwave, cold soak and freeze thaw maceration lowered the ratio 320/280 indicative of a lower proportion of skin derived caffeic acid in the wine. By contrast, both microwave and extended maceration improved wine hue associated with stable pigments (580/520). The resultant PQI for these was 38% higher than control wines, whereas PQI of freeze-thaw treated wines was 29% lower than control wines. Treatment with pectolytic enzyme or cold soaking did not significantly affect the PQI.

In wines made with added seed tannin the PQI was 20% lower than for control wines highlighting the significant impact of seed derived phenolics on the quality of the wine. By contrast, the addition of commercial skin tannin (20 mL/L) had no significant effect on PQI relative to the control wines.

Finally, the maceration treatments that involved cutting the berries (ACE) showed a blue shift in hue of stable (non-bleachable) pigments which had a significant and positive effect on the PQI (25 to 200% higher).

The outcome of this investigation is to recommend caution with respect to the selection of a maceration technique for Pinot Noir. One that limits the extraction of phenolic components from the seed is likely to be most beneficial to colour stability and also to the sensory properties of the wine as described in Chapter 7b.

Seed tannin has been associated with bitterness (Noble, 1998, Vidal et al., 2003a, Neves et al., 2010, McRae and Kennedy, 2011, Canuti et al., 2012) and has been attributed to the number of seeds in the berry rather than size of the seeds (Ristic and Iland, 2005). Bitterness has also been associated with gallic acid hich is in higher concentration in grape seeds than in grape skins (Paris, 1825, Yilmaz and Toledo, 2004). This may be due to the reaction of galloylated proanthocyanidins during winemaking as suggested by the results of earlier work by Neves et al. (2010) . A high concentration of seed tannin has also been

associated with a high concentration of free anthocyanin but a low concentration of pigmented tannins (Chapter 6a). By comparison, skin tannins have been associated with astringency (McRae et al., 2010, Ristic et al., 2010) and in Pinot Noir are generally found in higher concentrations in berries from low vigour vines (Cortell et al., 2005, Cortell and Kennedy, 2006, Cortell et al., 2008, Song et al., 2013). Consequently, optimising the extraction of skin tannin may lead to improvements in colour density and stability, but further research is required to evaluate the sensory properties of such wines.

8.5 CONCLUSION

This investigation highlights the consequences of different maceration techniques used during winemaking, and is particularly important for Pinot Noir table wines. As an aid to decision making during vinification, a procedure to monitor the extraction and interaction of phenolic compounds from grape tissues during winemaking and maturity has been identified and summarised with a phenolic quality index. The procedure requires samples analysed using existing rapid analytical techniques for wine colour and tannin to be interpreted at two additional wavelengths. These chemical assays are relatively simple and inexpensive and are analysed using a bench top UV-VIS spectrophotometer and can provide results within 4 hours of sampling. Consequently, the latest finding described in this study has the potential to provide winemakers with the opportunity to refine and direct the selection of maceration techniques used to make wine from a given parcel of fruit, in accordance with the fruit quality and the wine style preferred.

8.6 SUBSEQUENT RESEARCH FOCUS

The development of the Phenolic Quality Index has been made possible by detailed evaluation of Pinot Noir wine characteristics in response to the maceration technique used

to extract seed and skin tannin during vinification. Results described in Chapter 7 of this thesis showed that there was a strong correlation between the phenolic attributes of the wine determined by spectroscopy and the sensory characteristics of the wine. Further research is recommended to evaluate the application of PQI using other red wine varieties. The potential of this tool to be further developed as a quick and inexpensive method for examining the progress of phenolic development during vinification, is yet to be realised.

9

CONCLUSIONS AND RECOMMENDATIONS

9.1 BACKGROUND

The challenges associated with Pinot Noir winemaking largely revolve around the unique phenolic profile of this cultivar of *Vitis vinifera*. This study sought to gain a better understanding of the types of phenolic compounds that influence wine quality to inform winemakers how a consistently high quality product might be produced, especially in terms of colour stability. This is a recognised weakness in Pinot Noir wines. Phenolic compounds are mainly derived from the skin and seed of the grapes. However the nature and proportion of phenolic compounds that are contributed by each tissue type, and ultimately become incorporated into the wine, was largely unknown prior to this study.

There are several maceration techniques used which promote the extraction of pigment and tannins from the grape tissues into the wine matrix. The research objective was to differentiate their contribution in terms of phenolic quality parameters. The outcomes of phenolic extraction, subsequent interactions of phenolic compounds during vinification and bottle ageing with each maceration technique were examined. Preliminary trials suggested the need for early and greater extraction of skin tannins in fermentation. Recognising that commercial crushing produced grape skins in which the ratio of severed edge to surface area is low, lead to the investigation of the role of skin fragment size in phenolic extraction, which could be easily increased by cutting the skins. In addition, a simple analytic method was devised to differentiate skin and seed derived phenolic compounds providing a qualitative estimation of these compounds in samples collected throughout the vinification process.

9.2 SUMMARY OF RESULTS

9.2.1 Fermentation Volume Studies for Red Wine Experimentation (Chapter 2)

Experiments were conducted in fermentation vessels that covered the must weight range from 0.2 to 10 kg that were used in 15 subsequent experiments conducted in the course of this investigation; a 330 kg ferment from the same parcel of fruit was also compared. The results showed no correlations between the phenolic composition of the wine and either the must weight, vessel volume or the surface to volume ration of the fermenting must. Subsequently, ferments that required more intricate preparation could be confidently prepared and in some cases compared with those that were large enough to provide sufficient volume of wine for sensory analysis and industry wine tastings.

9.2.2 Effect of maceration treatment on Pinot Noir wine phenolic composition (Chapter 3)

The comparison of six maceration techniques commonly used in commercial practice showed that, while most promoted the extraction of colour pigments and tannins, there were differences in the quality of the tannins which subsequently affected colour stability as the wine aged. Thus the choice of maceration technique was shown to play a vital role in the extraction and stabilisation of Pinot Noir wine phenolics. Cold soaking and microwave thermovinification wines had heightened colour intensity and stability at 30 months bottle age, however the source of the tannins that combined with anthocyanins to promote the development of non-bleachable pigments was not clear. As a consequence a detailed investigation of the phenolic response of wines made from individual berry components was conducted.

9.2.3 The contribution of berry components to the phenolic composition of Pinot Noir wine (Chapter 4).

To amplify the role of each grape tissue component, wines were made using either individual berry components (skin, seed or pulp) or with a double complement of one of these berry components. The results demonstrated that seed tannin had a significant role in the formation of non-bleachable pigments in Pinot Noir. However these were not stable in the long term. Seed tannins are more strongly adsorbed by pulp cell wall material than are skin tannins, and consequently the composition of finished wines closely reflected the proportion of skin tannins in the must. When present in excess, seed tannin limited the formation of stable non-bleachable pigments. This led to the investigation of other sources of grape phenolic compounds, to understand the potential of readily accessible supplements to modify the phenolic profile of PN wines.

9.2.4 Source of supplementary tannins impacts the phenolic composition of Pinot Noir wines (Chapter 5).

The addition of exogenous grape solids was evaluated for their potential to improve the phenolic profile of Pinot Noir wines by contributing the preferred type of tannin. The choice of grape solids was made on the basis of those that are available in commercial wineries at the time that grapes for Pinot Noir table wine are processed. Fresh grape pomace or grape skins from Pinot Noir, Pinot Gris or Chardonnay grapes, Pinot Noir marc and a commercial Liquid Grape Skin Tannin extract were compared by adding a 20% portion of these to a base Pinot Noir grape must.

Results showed that significant benefits to both wine hue and colour stability were derived from the addition of fresh Pinot Noir grape skins, fermented Pinot Noir grape skins or the commercial Liquid Grape Skin Tannin extract. The addition of fermented Pinot Noir grape

marc was found to reduce the colour stability of the wine, and neither Pinot Gris nor Chardonnay grape tissues had a significant effect. The reduced colour stability was attributed to the concentration of seed tannins in the grape solids. The experiments that followed aimed to confirm the role of seed tannins in Pinot Noir wines either by adding seeds or seed-based oenotannins to the wine (Chapter 6a); or gradually removing the seeds from the fermenting must (Chapter 6b).

9.2.5 Seed tannin reduces colour stability in Pinot Noir wine (Chapter 6a).

Seed tannins, sourced either from fermented grape seed or a commercial seed oenotannin, when added to Pinot Noir musts increased both the tannin composition and brightness of the wine. The formation of stable pigments was limited while the concentration of free unstable anthocyanin pigments in the wine matrix increased. However, the nature of the colour pigments, as either free anthocyanins or anthocyanins bound to seed derived tannins, suggested structural vulnerability of the colour pigments, thereby limiting the ageing potential of the wine. The importance of skin tannins for the effective formation of stable pigments relative to seed tannins was confirmed.

9.2.6 Seed removal during fermentation improves the phenolic quality of Pinot Noir wine (Chapter 6b).

To be commercially applicable, the practicality of removing seeds from fermenting wine warrants serious consideration. This investigation explored the response of Pinot Noir wines to the gradual removal of seeds from the fermenting must with a view to selecting the optimum timing for such an operation in a commercial winery. The procedure resulted in a significant reduction in wine tannin, and had no detrimental effect on either wine colour density or the concentration of stable pigment. Histochemical studies showed that the tannin within the seed tissues moved from the outer integument of the seed to the

cells beneath the epidermis within 24 hours of yeast inoculation. The tannins were released slowly into the wine matrix as the cellular integrity of the seed coat declined. The optimum time to remove the seeds from the fermenting must was found to be 24 hours after inoculation. The problem of over-extraction of seed tannin in Pinot Noir may therefore be addressed by removing the seeds early in fermentation. However while this procedure may positively impact wine colour stability, it is unlikely to augment the colour intensity of the wine. Consequently an alternative way of approaching this issue was investigated which involved enhancing the extraction of pigment and tannin from the grape skins.

9.2.7 Accentuated Cut Edges (ACE): Reducing skin particle size, increases phenolic extraction from Pinot Noir grapes (Chapter 7a)

This work was divided into two parts, as sensory analysis of ACE macerated wines was conducted in the third year of the investigation.

In 2011, a technique for promoting the extraction of colour pigment and tannin from Pinot Noir grapes was explored which reduced the particle size of the grape pomace components. As the investigations progressed through 2012 and 2013, the focus shifted to using reduced particle size to enhance skin tannin extraction at the expense of seed tannins, with the aim of promoting wine colour density and stability. An additional benefit resulting from the improved extraction of grape skin phenolics, was the development of wines with superior hue (blue-purple pigments) which will further prolong the ageing potential of the wine.

9.2.8 Cutting Edge Pinot Noir: innovative maceration technique affects the phenolic, sensory and aroma characteristics of Pinot Noir wines (Chapter 7b)

ACE maceration was compared with the submerged cap maceration technique used in the earlier trials of this thesis. Submerged cap maceration involves holding the pomace cap below the surface of the fermenting must by placing a physical barrier on the top of the fresh must. The two maceration techniques were found to be strongly correlated with specific phenolic attributes determined by spectroscopy, and fruit flavours determined by aroma and full sensory analyses. Wines produced using submerged cap maceration had predominantly dark cherry aroma and flavours. By contrast wines made using ACE maceration had more intense red colouration and a higher proportion of tannin, stable colour pigments, fruit and floral aromas. The association of phenolic parameters determined by spectroscopy with sensory characteristics led to the search for definitive spectral properties of the wine that could be ascribed to either skin or seed derived components.

9.2.9 Development of a Phenolic Quality Index to differentiate seed and skin phenolics in Pinot Noir (Chapter 8).

The experiments described in this thesis utilised 20 different pomace manipulations and were designed to emphasise the relative contribution of grape skins and seeds to wine phenolic quality. The results ultimately led to the development of spectral profiles for each wine in which each tissue type could be distinguished. From these profiles and the spectral profiles of isolated grape and wine samples, a Phenolic Quality Index (PQI) was determined. The PQI uses an algorithm of three absorbance ratios from the ultra-violet-visible spectrum that have been reported to differentiate skin and seed derivatives. The

PQI is a simple analytical tool that can be used to monitor the phenolic quality of the wine during vinification.

9.3 IMPLICATIONS OF RESEARCH RESULTS

The phenolic evaluation of Pinot Noir wine was shown to be independent of vessel volume over the must weight range from 200 grams to 10 kilograms in a replicated trial and to an unreplicated lot of 330 kg, confirming that preliminary microvinification trials have a valuable role preceding full-scale industrial trials.

The choice of maceration technique used to promote colour and tannin extraction from Pinot Noir grapes was shown to have a significant impact on the ageing potential of the wine. A range of maceration techniques or the addition of oenotannins, have traditionally been used in the production of Pinot Noir wines. This research investigation suggests closer scrutiny of the tannin source either extracted from the must or added to the wine is recommended. Higher tannin concentrations *per se* may benefit wine astringency and palate length, but were found to have conflicting effects on the formation of stable colour pigments. Skin tannins were found to be more beneficial to the development of stable wine colour than were seed tannins.

The availability of exogenous sources of grape solids in most wineries may be viewed as a valuable alternative to oenological tannin additions for supplementing wine tannin. However the experiments conducted during the course of this investigation showed that, while exogenous grape solids may be beneficial in some instances, their inclusion in grape must should be handled with caution and regular monitoring of the phenolic status of the wine as fermentation progresses would be conducive to fine-tuning the quality of the developing wine.

In order to make a preliminary evaluation of the ACE maceration technique developed during the course of this investigation, seeds were removed from the fermenting must, with only those seeds that had fallen to the base of the fermentation vessel being selected. While seed removal was demonstrated to improve phenolic quality in Pinot Noir wines, the proportion of seeds that might be readily removed during early fermentation in commercial wineries remains problematic.

As a consequence of the research, two techniques with potential for commercial application were identified:

1. ACE maceration: a technique that can be applied prior to fermentation to promote the extraction and development of stable colour pigments.
2. Phenolic Quality Index: an analytical tool by which the phenolic attributes of the grapes and wine can be differentiated using spectral analysis.

Sensory evaluation of ACE macerated wines showed an increase in fruit and floral aromas and flavours, however astringency and bitter flavours were also identified. The bitter flavour attribute is most likely to be caused by greater extraction of phenolic substances from the seeds as they are freed from the pulp tissues. Skin tannins have been associated with astringency due to their molecular size and configuration and their extraction is likely to be enhanced where skin tannin extraction is amplified. Pressing the wine off from the grape pomace soon after ACE maceration has the potential to reduce the impact of phenolic components extracted from seed while optimising extraction of skin tannins and colour pigments. In addition, ACE maceration followed by early press off would reduce the tank space required to complete fermentation thereby increasing winery throughput. Early press off opens the opportunity for barrel fermentation as with premium whites, and the option to ferment at lower temperatures, thereby preserving volatile aroma

compounds. ACE maceration and early press off may also benefit other red wine varieties and is an area worthy of further investigation.

The Phenolic Quality Index (PQI) determined as a consequence of these investigations was calculated following two simple chemical assays, which can be completed within 4 hours of sampling the must or wine. The PQI calculation provides a convenient method for monitoring the phenolic composition of the wine as it ferments, and also for assessing the ageing potential of finished wines. In practice, the PQI has the potential to provide winemakers with the opportunity to further refine and even redirect the selection of maceration techniques used to make wine from a given parcel of fruit, in accordance with the fruit quality and the preferred wine style. For example, regular testing of PQI at a particular winery would generate a database of PQI values in response to changes in fruit quality, maceration technique and grape variety. Collected over a number of vintages the PQI can be calculated as a routine test and this pool of data used as a reference tool to direct winemaking procedures in response to vintage variability.

9.4 RECOMMENDATIONS

- The assessment of fruit quality at harvest should inform the choice of maceration technique used in vinification of Pinot Noir table wine.
- The preferential extraction of skin and seed tannins imposed by the maceration technique selected during the winemaking should be closely monitored during the vinification process.
- Maceration techniques that optimise extraction of skin tannins should be strongly considered when making table wine made from Pinot Noir.

- The application of ACE maceration to increase turnover of wine processing operations for a range of red wine varieties.
- The phenolic quality (PQI) should be promoted as both a research and commercial management tool.

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